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(57) Abstract

The present invention relates to novel peptides that are potent cytokine regulatory agents. In addition, the present invention relates to pharmaceutical compositions comprising a pharmaceutically acceptable carrier and a cytokine regulatory agent. Administration of such a cytokine regulatory agent to a subject can enhance or restrain cytokine activity. Thus, the present invention provides a method of regulating cytokine activity in a subject having a condition characterized by aberrant or altered cytokine activity. The invention also provides methods of treating such conditions, including, for example, disuse deconditioning, diseases mediated by nitric oxide and cytokines, adverse drug reactions, obesity, septic shock, and adverse side effects due to cancer chemotherapy or occurring as in response to organ transplantation.

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CYTOKINE REGULATORY AGENTS AND METHODS OF USE IN PATHOLOGIES AND CONDITIONS ASSOCIATED WITH ALTERED CYTOKINE LEVELS

BACKGROUND OF THE INVENTION

5   This application is a continuation-in-part of application Serial No. 08/484,262, filed June 7, 1995, which is a continuation-in-part of application Serial No. 08/400,983, filed March 6, 1995, each of which is incorporated herein by reference in its entirety.

10

FIELD OF THE INVENTION

The present invention relates generally to the fields of peptide chemistry and molecular pathology and, more specifically, to novel cytokine regulatory agents

15   and their use in controlling cytokine-regulated physiologic processes and pathologies.

BACKGROUND INFORMATION

Cytokines are a class of secreted, soluble proteins produced by a variety of cells in response to

20   many different kinds of inducing stimuli, including environmental, mechanical, and pathological stresses. Lymphoid, inflammatory and hemopoietic cells secrete a variety of cytokines which regulate the immune response by controlling cell proliferation, differentiation and

25   effector functions. For example, regulatory cytokines produced in response to T cell stimulation during an immune response can be immunosuppressive or immunostimulatory. The immune response and acute phase

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response associated with altered cytokine levels can occur, for example, due to disuse deconditioning, organ damage such as that associated with transplantation, cancer treatment, septic shock and other bacterially

5   related pathologies, adverse drug reactions, nitric oxide mediated tissue damage and diabetes.

Cytokines are normally present in very low concentrations in a tissue and their effects are mediated through binding to high affinity receptors on specific

10   cell types. Various cytokines such as the interleukins (IL), interferons (IFN), colony stimulating factors (CSF) and tumor necrosis factors (TNF) are produced during immune, inflammatory, repair and acute phase responses and they control various aspects of these responses.

15   Following induction of such an immune, inflammatory, repair or acute phase response, the concentrations of various cytokines can increase or decrease at different times. For example, increased levels of cytokines are associated with a variety of situations such as space

20   flight, immobilization, spinal cord injury, and bed rest, which result in disuse deconditioning. During space flight, for example, TNF, IL-6, and IL-2 levels increase upon a subject's initial exposure to weightlessness and again upon return from space.

25   Altered levels of cytokines have also been linked to abnormal bone metabolism and the rapid decalcification that occurs during immobilization, spinal cord injury, or long-term bed rest. Similarly, cytokine levels are altered during chronic states such as during

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repair and autoimmune reactions to organ damage,
nephrotoxicity associated with the administration of
cyclosporine to transplant subjects, cancer chemotherapy,
as well as in individuals that are obese or suffering
5   from diabetes, septic (endotoxic) shock or
glomerulonephritis.

Cytokines, including the TNFs, CSFs,
interferons and interleukins mediate host defense
responses, cell regulation and cell differentiation. For
10   example, these cytokines can induce fever in a subject,
can cause activation of T cells, B cells and macrophages,
and can affect the levels of other cytokines, which
result in a cascade effect whereby other cytokines
mediate the biological levels and actions of the first
15   cytokine.

Cytokines may regulate the immune response
through immunostimulatory or immunosuppresive effects.
For example, IL-10 can block activation of many of the
inflammatory cytokines including TNF, IL-1 and IL-6,
20   while upregulating anti-inflammatory cytokines, such as
IL-12. IL-10, which is produced by macrophages and other
cell types, also stimulates the proliferation of mast
cells and thymocytes and inhibits various functions of
monocytes and macrophages. As a consequence of this
25   monocyte and macrophage inhibition, the activity of T
cells is also affected. The full scope of the role of
IL-10 in the immune system is only beginning to be
understood.

Cytokines have multiple biological activities and interact with more than one cell type. Thus, it has not been possible to target one particular cytokine or cell type to prevent the damaging side effects of

5   treatment. A better approach for preventing damage due to the unwanted and uncontrolled over-suppression or over-stimulation of cytokine activity would be to regulate the expression of the relevant or controlling cytokine or cytokines involved in an immune response

10 without eliminating or over-expressing any one cytokine. Such a treatment would not create or aggravate a pathological or ongoing immune response. In this way, pathological immune-mediated effects, such as immunosuppression or autoimmune reactions, can be

15 prevented and homeostasis can be maintained.

Corticosteroids can be used to modulate cytokine expression. However, they can cause complete immunosuppression and have other undesirable side effects, such as inducing "wasting" syndrome, diabetes

20 and osteoporosis. Similarly, non-steroidal anti-inflammatory drugs (NSAID), such as ketorolac (Toradol®; Syntex), are effective in treating inflammation and pain. However, NSAIDs also cause undesirable side effects by inhibiting prostaglandin production, which can lead to

25 potentially severe complications including gastric ulceration, bleeding and renal failure.

In order to prevent pathological conditions or disruption of normal immune mediated functions caused by the aberrant expression of cytokines as described above,

it would be advantageous if cytokine levels could be accurately manipulated and efficaciously controlled. Thus, a need exists for agents that can regulate the activity of cytokines in a subject without causing

5   undesirable side effects. Furthermore, a need exists for identifying agents which can be used in the treatment of pathologies and conditions associated with altered cytokine levels. The present invention satisfies these needs and provides related advantages as well.

10   **SUMMARY OF THE INVENTION**

The present invention relates to novel peptides that are potent cytokine regulatory agents. As disclosed herein, such cytokine regulatory agents have the general structures, X_1 - X_2 - His - (D)Phe - Arg - (D)Trp - X_3,

15 and X_4 - X_5 - (D)Phe - Arg - (D)Trp - X_3, where X_1, X_2, X_3, X_4, and X_5 can be amino acids or amino acid analogs. The invention also relates to a cytokine regulatory agent having the structure, Ac-His-(D)Phe-Arg-{(D)Trp(CH$_2$)}-(NAc)Gly-NH$_2$, which contains a reduced (D)Trp analog.

20   In addition, the present invention relates to pharmaceutical compositions comprising a pharmaceutically acceptable carrier and a cytokine regulatory agent or agents. Administration of such a cytokine regulatory agent or a combination thereof to a subject can modify

25 the levels of various cytokines in the individual, which will result in immunostimulation or immunosuppression depending on the particular cytokines that are regulated.

The present invention also provides methods of regulating through enhancement the cytokine activity in a subject and methods of treating a condition, pathology or an injury characterized, in part, by altered or aberrant

5   cytokine activity. Such conditions, pathologies or injuries include disuse deconditioning, diseases mediated by nitric oxide and cytokines, diabetes, obesity, autoimmune diseases, septic (endotoxic) shock, glomerulonephritis, organ damage such as that which

10   occurs during transplantation and adverse side effects of cancer chemotherapy, such as nephrotoxicity.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1a and 1b demonstrate that a cytokine regulatory agent effectively restrains the decrease in

15   glomerular filtration rate that occurs due to cyclosporine-induced nephrotoxicity.

Figure 2 demonstrates that EX-2 enhances IL-10 levels in mouse plasma.

Figures 3a and 3b demonstrate that EX-2 induces

20   IL-10 levels in human plasma.

Figure 4 shows the body weights of obese (fa/fa) and normal (Fa/+) rats treated with EX-2.

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DETAILED DESCRIPTION OF THE INVENTION

The present invention generally relates to
novel cytokine regulatory agents having the structure:
X_1 - X_2 -His - (D)Phe - Arg - (D)Trp - X_3, wherein
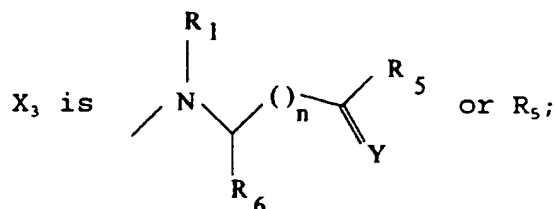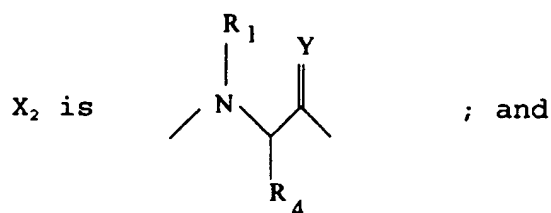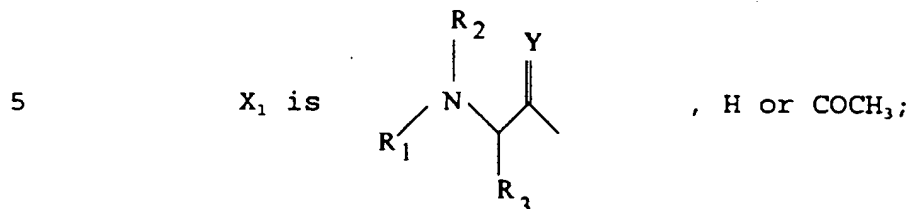
5   X_1 is

, H or $COCH_3$;

X_2 is

; and

X_3 is

or R_5;

wherein Y is O, H_2 or S; R_1 is H, $COCH_3$, C_2H_5, CH_2Ph, $COPh$,
COO-t-butyl, $COOCH_2Ph$, CH_2CO-(polyethylene glycol) or A;
10   R_2 is H or $COCH_3$; R_3 is a linear or branched alkyl group
having 1 to 6 carbon atoms or a cyclic alkyl group having
3 to 6 carbon atoms; R_4 is $(CH_2)_m$-$CONH_2$, $(CH_2)_m$-CONHR. or
$(CH_2)_m$-CONHA; R_5 is OH, OR_3, NH_2, SH, $NHCH_3$, $NHCH_2Ph$ or A;
and R_6 is H or R_3; and wherein "Ph" is C_6H_5; "m" is 1, 2 or

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3; "n" is 0, 1, 2 or 3; and "A" is a carbohydrate having the general formula:

$$\text{R}_1\text{O}\diagdown\overset{\displaystyle \text{O}}{\diagup}\overset{\text{NHR}_1}{\diagdown}\text{OR}_1$$
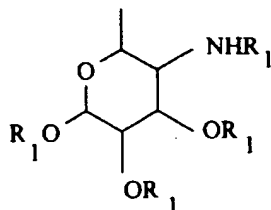
In one embodiment of the present invention, the
5   peptides have any of the variables described above for X_1 and X_2 and specifically have OH for X_3 (X_3 is R_5, wherein R_5 is OH). In yet another embodiment, Y, R_1, R_2, R_4, R_5, and R_6 can be any of the variables described above and R_3 is a cyclic alkyl group having 3 to 6 carbon atoms.

10   Exemplary peptides encompassed by the formulas disclosed above include Nle-Gln-His-(D)Phe-Arg-(D)Trp-Gly-NH_2; Ac-Nle-Gln-His-(D)Phe-Arg-(D)Trp-Gly-NH_2; and Ac-(cyclohexyl)Gly-Gln-His-(D)Phe-Arg-(D)Trp-Gly-NH_2, each of which can regulate cytokine activity.

15   The present invention also relates to novel cytokine regulatory agents having the structure:
X_4 - X_5 - (D)Phe - Arg - (D)Trp - X_3, wherein
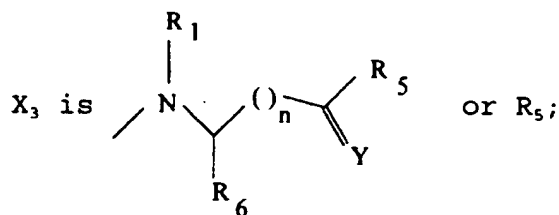
X_4 is $\quad$, H, $COCH_3$, or absent;

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X_5 is His, H, or $COCH_3$; and

X_3 is

$$\underset{R_6}{\overset{R_1}{N}} \quad \overset{()_n}{\longrightarrow} \underset{Y}{\overset{R_5}{=}} \quad \text{or } R_5;$$

wherein Y is O, H_2 or S; R_1 is H, $COCH_3$, C_2H_5, CH_2Ph, COPh, COO-t-butyl, $COOCH_2Ph$, CH_2CO-(polyethylene glycol) or A;

5   R_2 is H or $COCH_3$; R_4 is $(CH_2)_m$-$CONH_2$, $(CH_2)_m$-$CONHR_1$ or $(CH_2)_m$-CONHA; R_5 is OH, OR_3,, NH_2, SH, $NHCH_3$, $NHCH_2Ph$ or A; and R_6 is H or R_3; and wherein "Ph" is C_6H_5; "m" is 1, 2 or 3; "n" is 0, 1, 2 or 3; and "A" is a carbohydrate having the general formula
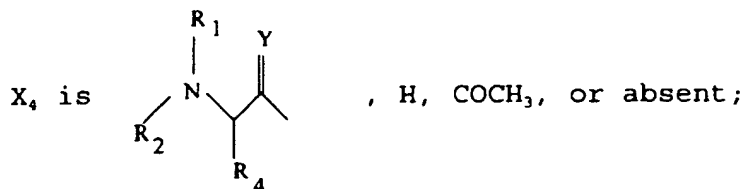
$$\underset{R_1O}{} \overset{O}{\longrightarrow} \underset{OR_1}{\overset{NHR_1}{\longrightarrow}} OR_1 \quad .$$
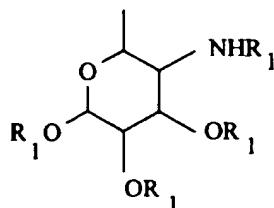
10

In one embodiment of the present invention, the peptides are shortened analogs of 3 to 5 amino acid residues, having any of the variables described above for X_4 and X_5 and specifically having OH for X_3 (X_3 is R_5,

15   wherein R_5 is OH). In yet another embodiment, where X_4 is absent, X_5 is H or $COCH_3$, and X_3 is R_5, the cytokine regulatory agents are tripeptides of (D)Phe-Arg-(D)Trp. Preferred peptides include His-(D)Phe-Arg-(D)Trp-Gly-NH_2; Ac-His-(D)Phe-Arg-(D)Trp-NH_2; His-(D)Phe-Arg-(D)Trp-OH;

10

Ac-(D)Phe-Arg-Trp-NH$_2$; and cyclo(His-(D)Phe-Arg-(D)Trp),
which can regulate cytokine activity.

5   As used herein, the terms "regulate" or
"regulatory" mean to control by enhancing, limiting,
restricting, restraining, modulating or moderating. Such
regulation includes the pleiotropic, redundant,
synergistic or antagonistic effects that occur due to the
activity of biological agents such as cytokines, which
10   can affect a variety of biological functions directly or
indirectly through cascade or biofeedback mechanisms.

       As used herein, the term "cytokine regulatory
agent" means an agent that controls cytokine activity by
enhancing, limiting, restricting, restraining, modulating
15   or moderating the biological activity of a cytokine. It
should be recognized, however, that while the cytokine
regulating agents generally can regulate cytokine
activity, no specific mechanism of action is proposed as
to how a cytokine regulatory agent acts to effect a
20   condition characterized by altered or aberrant cytokine
activity.

       Cytokines are well known in the art and
include, but are not limited to the tumor necrosis
factors (TNFs), colony stimulating factors (CSFs),
25   interferons (INFs), interleukins (IL-1, IL-2, IL-3, IL-4,
IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13,
IL-14, and IL-15), transforming growth factors (TGFs),
oncostatin M (OSM), leukemia inhibiting factor (LIF),
platelet activating factor (PAF) and other soluble

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immunoregulatory peptides that mediate host defense
responses, cell regulation and cell differentiation (see,
for example, Kuby, Immunology 2d ed. (W.H. Freeman and
Co. 1994); see Chapter 13, which is incorporated herein
5   by reference).

        The cytokine regulatory agents of the invention
can regulate the aberrant or altered expression of one or
more cytokines that occurs in various conditions,
including, for example, pathologies, immune responses and
10 inflammatory responses. Such conditions are considered
together for purposes of the present invention is that
they are characterized, in part, by altered or aberrant
cytokine activity and, therefore, are amenable to
regulation by one or more cytokine regulatory agents.

15   As used herein, the term "characterized by"
means contributes or affects, at least in part. Though
cytokine contribution can be, it does not have to be, and
the only, primary, or even a major factor of the
condition. For example, it is well understood in the art
20 that an infection has altered cytokine levels and is,
therefore, a condition characterized by cytokine
activity, but that cytokines activity is only a part of
the infectious condition.

        As used herein, the term "condition
25 characterized by altered or aberrant cytokine activity"
includes all cytokine regulated or modulated pathologies
and injuries, including the immune, inflammatory and
healing processes associated with an injury. The skilled

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artisan can recognize such a condition by detecting an increased or decreased level or activity of a particular cytokine as compared to the normal level of the cytokine expected to be found in a healthy individual. Methods
5   for determining such normal levels are well known in the art.

Conditions characterized by altered or aberrant cytokine activity include, but are not limited to, disuse deconditioning, organ damage such as occurs in response
10 to organ transplantation; adverse reactions associated with cancer chemotherapy; obesity; diseases such as diabetes and atherosclerosis that are mediated by free radicals and nitric oxide action; bacterial endotoxic sepsis and related shock; pain; cachexia; adult
15 respiratory distress syndrome; and autoimmune or other patho-immunogenic diseases or reactions such as allergic reactions or anaphylaxis, arthritis, inflammatory bowel disease, glomerulonephritis, systemic lupus erythematosus, transplant atherosclerosis and parasitic
20 mediated immune dysfunctions such as Chagas' Disease.

A cytokine regulatory agent can be, for example, a peptide comprising amino acids or amino acid analogs as described herein. In addition to the examples provided above, other representative examples of peptide
25 cytokine regulatory agents include:

1) Ac-Nle - Gln - His - (D)Phe - Arg - (D)Trp - Gly-OH;

2) Ac-Nle - Gln - His - (D)Phe - Arg - (D)Trp - Gly-NH$_2$;

3) Ac-Nle - Gln - His - (D)Phe - Arg - (D)Trp - Gly-OC$_2$H$_5$;

4) Ac-Nle - Gln - His - (D)Phe - Arg - (D)Trp -

Gly-NH-NH$_2$;

5) Ac-Nle - Asn - His - (D)Phe - Arg - (D)Trp - Gly-NH$_2$;

6) Ac-Nle - Asn - His - (D)Phe - Arg - (D)Trp - Gly-OH;

7) Ac-Nle - Gln - His - (D)Phe - Arg - (D)Trp - Gly-

5   NHCH$_2$CH$_2$Ph;

8) Ac-Nle - Gln - His - (D)Phe - Arg - (D)Trp - Gly-

NHCH$_2$Ph;

9) Nle - Gln - His - (D)Phe - Arg - (D)Trp - Gly

10   N─────────────────────────────────── O;

10) Ac-Gln - His - (D)Phe - Arg - (D)Trp - Gly-NH$_2$;

11) Ac-Nle - Gln - His - (D)Phe - Arg - (D)Trp-NH$_2$;

12) Ac-His - (D)Phe - Arg -(D)Trp - Gly-NH$_2$;

13) His - (D)Phe - Arg - (D)Trp-NH$_2$;

15 14) Ac-His - (D)Phe - Arg - (D)Trp-OH; and

15) Ac-His - (D)Phe - Arg - {(D)Trp(CH$_2$)} - (NAc)Gly-NH$_2$,

wherein {(D)Trp(CH$_2$)} is an analog of (D)Trp, where H$_2$

replaces the α-carbonyl oxygen and (NAc)Gly is a glycine

derivative with an N-acetyl moiety.

20   Peptide cytokine regulatory agents as described

above are characterized, in part, by a core structure

having the amino acid sequence, (D)Phe - Arg - (D)Trp, or

an analog of (D)Trp, and more preferably the core amino

acid sequence His-(D)Phe-Arg-(D)Trp, or an analog of

25 (D)Trp. The amino acids are indicated by their commonly

known three letter code and (D) designates an amino acid

having the "D" configuration, as opposed to the naturally

occurring L-amino acids. Where no specific configuration

is indicated, one skilled in the art would understand the

30 amino acid to be an (L)-amino acid. In the peptides

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exemplified above, "Nle" is the three letter code for norleucine and "Ph" indicates a "phenyl" group (C_6H_5).

Cytokine regulatory agents such as the peptides described above were synthesized using a modification of
5   the solid phase peptide synthesis method of Merrifield (J. Am. Chem. Soc., 85:2149 (1964), which is incorporated herein by reference) or can be synthesized using standard solution methods well known in the art (see, for example, Bodanzsky, M., Principles of Peptide Synthesis 2nd
10 revised ed. (Springer-Verlag, 1988 and 1993), which is incorporated herein by reference). Peptides prepared by the method of Merrifield can be synthesized using an automated peptide synthesizer such as the Applied Biosystems 431A-01 Peptide Synthesizer (Mountain View,
15 CA) or using the manual peptide synthesis technique described by Houghten, Proc. Natl. Acad. Sci., USA 82:5131 (1985), which is incorporated herein by reference.

Peptides were synthesized using amino acids or
20 amino acid analogs, the active groups of which were protected as necessary using, for example, a t-butyldicarbonate (t-BOC) group or a fluorenylmethoxy carbonyl (FMOC) group. Amino acids and amino acid analogs can be purchased commercially (Sigma Chemical
25 Co.; Advanced Chemtec) or synthesized using methods known in the art. Peptides synthesized using the solid phase method can be attached to resins including 4-methylbenzhydrylamine (MBHA), 4-(oxymethyl)-phenylacetamido methyl and 4-(hydroxymethyl)phenoxy

methyl-copoly(styrene-1% divinylbenzene) (Wang resin),
all of which are commercially available, or to
p-nitrobenzophenone oxime polymer (oxime resin), which
can be synthesized as described by De Grado and Kaiser,
5   _J. Org. Chem._ 47:3258 (1982), which is incorporated
herein by reference.

    One skilled in the art would know that the
choice of amino acids or amino acid analogs incorporated
into the peptide will depend, in part, on the specific
10 physical, chemical or biological characteristics required
of the cytokine regulatory agent. Such characteristics
are determined, in part, by the route by which the
cytokine regulatory agent will be administered or the
location in a subject to which the activity of the   ·
15 cytokine regulatory agent will be directed.

    Selective modification of the reactive groups
in a peptide also can impart additional desirable
characteristics to a cytokine regulatory agent. Peptides
can be manipulated while still attached to the resin to
20 obtain N-terminal modified compounds such as an
acetylated peptide or can be removed from the resin using
hydrogen fluoride or an equivalent cleaving reagent and
then modified. Compounds synthesized containing the
C-terminal carboxy group (Wang resin) can be modified
25 after cleavage from the resin or, in some cases, prior to
solution phase synthesis. Methods for modifying the
N-terminus or C-terminus of a peptide are well known in
the art and include, for example, methods for acetylation
of the N-terminus or methods for amidation of the

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C-terminus. Similarly, methods for modifying side chains
of the amino acids or amino acid analogs are well known
to those skilled in the art of peptide synthesis. The
choice of modifications made to the reactive groups
5   present on the peptide will be determined by the
characteristics that the skilled artisan requires in the
cytokine regulatory agent.

Particular cyclic peptides also can be an
effective cytokine regulatory agent. A cyclic peptide
10   can be obtained by inducing the formation of a covalent
bond between, for example, the amino group at the
N-terminus of the peptide and the carboxyl group at the
C-terminus. For example, the peptide, cyclo(His-(D)Phe-
Arg-(D)Trp), which can be produced by inducing the
15   formation of a covalent bond between His and (D)Trp, can
have cytokine regulatory activity. Alternatively, a
cyclic peptide can be obtained by forming a covalent bond
between a terminal reactive group and a reactive amino
acid side chain or between two reactive amino acid side
20   chains. One skilled in the art would know that the
choice of a particular cyclic peptide is determined by
the reactive groups present on the peptide as well as the
desired characteristic of the peptide. For example, a
cyclic peptide may provide a cytokine regulatory agent
25   with increased stability, increased solubility, decreased
immunogenicity, or decreased clearance *in vivo*.

A newly synthesized peptide can be purified
using a method such as reverse phase high performance
liquid chromatography (RP-HPLC), which is described in

detail below (see Example I), or other methods of
separation based on the size or charge of the peptide.
Furthermore, the purified peptide can be characterized
using these and other well known methods such as amino

5   acid analysis and mass spectroscopy, which are described
in detail below (see Example I).

The invention also relates to pharmaceutical
compositions comprising a cytokine regulatory agent or
agents and a pharmaceutically acceptable carrier.

10 Pharmaceutically acceptable carriers are well known in
the art and include aqueous solutions such as
physiologically buffered saline or other buffers or
solvents or vehicles such as glycols, glycerol, oils such
as olive oil or injectable organic esters.

15   A pharmaceutically acceptable carrier can
contain physiologically acceptable compounds that act,
for example, to stabilize the cytokine regulatory agent
or increase the absorption of the agent. Such
physiologically acceptable compounds include, for

20 example, carbohydrates, such as glucose, sucrose or
dextrans, antioxidants, such as ascorbic acid or
glutathione, chelating agents, low molecular weight
proteins or other stabilizers or excipients. One skilled
in the art would know that the choice of a

25 pharmaceutically acceptable carrier, including a
physiologically acceptable compound, depends, for
example, on the route of administration of the cytokine
regulatory agent and on the particular physio-chemical

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characteristics of the specific cytokine regulatory
agent.

A cytokine regulatory agent as described above
or a pharmaceutical composition containing a cytokine
5   regulatory agent can be administered to a subject in
order to regulate pathologically modified, including
depressed or elevated, cytokine activity in the subject.
For example, the peptide or composition can be
administered to a subject as a treatment for traumatic
10   injuries, bacterial sepsis and endotoxic shock,
inflammation, pain, cachexia, adult respiratory distress
syndrome, transplant atherosclerosis, and patho-
immunogenic diseases such as arthritis, inflammatory
bowel disease systemic lupus erythematosus and other
15   autoimmune dysfunctions, each of which is characterized
by pathologically elevated inflammatory cytokine
activity.

As used herein, the term "pathologically
elevated" means that a cytokine activity is elevated
20   above a range of activities which is expected in a normal
population of such subjects and which is associated with
a pathological response. For example, a normal range of
IL-1, such as IL-1β, activity present in a specific
tissue can be determined by sampling a number of subjects
25   in the population. A subject having a pathology
characterized by cytokine-induced pathological effects
can be readily identified by determining that the
cytokine activity in the subject is pathologically

elevated, which is above the normal range and which
results in a pathological response such as a fever.

     In addition, as described in more detail below,
the peptides or pharmaceutical compositions can also be
5   used for treating obesity, disuse deconditioning and
diseases mediated by nitric oxide, such as diabetes and
glomerulonephritis, transplant atherosclerosis, multiple
sclerosis, as well as for reducing the negative effects
of a cancer chemotherapeutic agent, and for protecting
10   organs against damage, induced by chemotherapeutic,
antifungal (amphotericin), antibacterial (aminoglycoside
antibiotics), and antiviral (azidothymidine) agents, all
of which are associated with pathologically altered
levels of cytokines.

15   Disuse Deconditioning

     Altered levels of cytokines are associated with
a variety of conditions that lead to disuse
deconditioning, such as space flight, immobilization,
spinal cord injury, bed rest, and trauma. Examples of
20   other conditions which result in disuse deconditioning
include, for instance, simulated weightlessness, casting,
and denervation.

     Space flight, for example, alters many immune
responses and regulatory components of the immune
25   response, including the cytokine network. Space flight,
as well as model systems of space flight, affect the
production and activation of various cytokines including

20

interleukins and TNF. Levels of IL-6 and IL-2 are
elevated on the first day of space flight and again after
returning from space flight.

      Cytokines also modulate the function of
5 osteoclasts, and IL-1 and TNF have been implicated in the
rapid decalcification or loss of bone mineral density
that results from immobilization, bed rest and spinal
cord injury. Correlation between cytokine expression and
increased retrograde transport following injury suggests
10 that cytokines play a role in peripheral nerve damage.
Bed rest, when combined with intra-articular
administration of steroids, produces a particularly rapid
systemic effect on the acute phase response that is
mediated by cytokines.

15   A cytokine regulatory agent of the present
invention or a composition containing the peptide can be
used for treating disuse deconditioning by reducing or
ameliorating the negative effects of disuse
deconditioning associated with altered cytokine levels.
20 The negative effects include, for example, loss of muscle
mass, bone density, exercise capacity, and oxygen
consumption as well as decreased levels of oxidative and
antioxidant enzymes, such as soleus citrate synthase, in
muscle tissue.

Organ Protection

Cytokines also play an important role in organ
damage and organ protection. For example, cytokines
significantly affect such events and conditions as organ
5   transplant, particularly the rejection of a transplanted
organ, transplant atherosclerosis, ischemia-reperfusion,
cyclosporine-induced nephrotoxicity, myocardial
infarction, and stroke.

Regarding organ transplant, cytokines,
10   especially tumor necrosis factor (TNF), are important
mediators of allograft rejection. Elevated serum levels
of TNF occur in patients undergoing renal, hepatic, and
cardiac allograft rejection. Further evidence of TNF
presence has been demonstrated in rejecting rat cardiac
15   and renal allografts as well as human renal and hepatic
grafts. Acute rejection episodes in renal transplant
patients are accompanied by an increase in serum IL-6
levels. The administration of glucocorticoids during
such episodes was reported by Ray et al., J. Am. Soc.
20   Nephr. 2(12):5214-5221 (1992), to lead to a rapid and
marked decrease in IL-6 levels and the severity of the
rejection episodes. The cytokine regulatory agents of
the present invention can be similarly useful in reducing
the severity of such rejection episodes and the damage
25   caused thereby.

Administration of immunosuppressive agents,
such as cyclosporine, to transplant subjects also effects

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cytokine activity, causing cyclosporine-induced
nephrotoxicity resulting in damage to the kidney. Agents
which regulate cytokines, such as those of the present
invention, can be effective in reducing cyclosporine-
5   induced nephrotoxicity.

In addition, cytokine regulatory agents of the
present invention can protect against organ damage
associated with ischemia-reperfusion, including such
damage as decreased glomerular filtration rate and
10 increased vascular resistance in the kidney. Ischemia-
reperfusion injury has also been associated with
elevations of cytokines. IL-1 and TNF mediate myocardial
ischemia and increased levels of IL-1 can occur during
the reperfusion phase of skeletal muscle ischemia.
15 Increased TNF release follows both moderate and severe
ischemic injury to the liver.

Thus, the present invention provides a method
of protecting an organ of a subject against organ damage
by administering a cytokine regulatory agent to the
20 subject. The organ damage to be protected against can be
the result of such conditions or events as an organ
transplant, the administration of an immunosuppressive
agent, such as cyclosporine, the resulting damage from
ischemia-reperfusion, myocardial infarction, stroke, and
25 the like.

As used herein "damage" includes damage to a
whole organ, such as the dysfunctioning of an organ, or
damage to a tissue of the organ. In many instances, such

damage involves, not only increased cytokine levels, but also increases in free radicals. For example, the activated white blood cells that result from ischemia release oxygen radicals and deliver aggressive mediators

5   such as cytokines and PAF, which are chemotactic for leukocytes. This results in a cycle in which oxygen radicals and mediators, such as cytokines, are responsible for the augmentation of post-ischemic damage.

The type of damage which free radicals or
10   cytokines can induce includes, but is not limited to, inflammation, atherosclerosis, increases in vascular permeability, fibrosis, and necrosis. These and other types of damage can be assessed histomorphologically by methods well known in the art. Damage also can be
15   evidenced by organ dysfunction that is often reflected by changes in physiological and biochemical parameters which tend to be specific to a particular organ or tissue and which can be routinely measured by techniques well known in the art. For example, kidney dysfunction, indicative
20   of renal damage, might lead to decreases in glomerular filtration rate and renal blood flow as well as increased renal vascular resistance. Examples of biochemical parameters that can be used as early markers of tissue and organ damage include increased nitric oxide or
25   malondialdehyde levels. Malondialdehyde is a byproduct of lipid peroxidation and, therefore, indicative of free radical levels. Alternatively, or in addition thereto, increased levels of such enzymes as lactate dehydrogenase, a marker of cell death, can be assessed.

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Organs which can be protected against damage by administration of the cytokine regulatory agents of the present invention include, but are not limited to, the heart, kidney, liver, lung, brain, muscle, skin, or bone.

5   An example of tissue within an organ which can be protected by the present invention is the epithelium in skin. Also included are organs which can be transplanted or which are associated with the negative side effects caused by the administration of an immunosuppressive

10 agent to a transplant subject, as well as organs subject to ischemia-reperfusion, infarction, stroke, or other specific conditions or events which might lead to cellular damage or death. More than one organ may be protected at a time.

15   Cancer Chemotherapy

A cytokine regulatory agent of the present invention, or composition containing the agent, can also be used in cancer chemotherapy for reducing the nephrotoxic effect or other negative effects of cancer

20 chemotherapeutic agents.

Cancer chemotherapeutic agents, such as cisplatin, Taxol* and Adriamycin*, induce and enhance the production of cytokines. Taxol*, a microtubule-

25 stabilizing anti-neoplastic agent, induces expression of TNF and IL-1 and enhances the production of TNF in macrophages. Increased concentrations of IL-6 and TNF occur in patients receiving cisplatin, with cytokine concentrations being particularly elevated in patients

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experiencing renal or hepatic toxicity. Cytokine
production is also increased in cancer patients during
chemo-hyperthermia. TNF and IL-6 levels measured when a
hyperthermia-inducing heating solution containing
5 cisplatin was administered showed a dramatic increase in
IL-6 occurring within 30 minutes after the treatment
started. TNF values were only slightly elevated.

As shown in the Examples, use of a cytokine
regulatory agent of the invention in cancer chemotherapy
10 can reduce the nephrotoxic effect of a cancer
chemotherapeutic agent. Moreover, the peptides can be
administered to a subject undergoing cancer chemotherapy
to reduce other negative side effects of chemotherapeutic
drugs, including but not limited to, nausea, vomiting,
15 mucositis, anorexia, fatigue, and other organ
dysfunctions.

Diseases Mediated By Nitric Oxide and Cytokines, Including Diabetes and Glomerulonephritis

Cytokines and nitric oxide (NO) are both
20 important mediators of a variety of disease states,
including, for example, diabetes and glomerulonephritis.
Cytokines and NO regulate beta-cell damage in early
insulin-dependent diabetes mellitus. IL-1, interferon
gamma and TNF induce islet NO. In parallel with NO
25 production, IL-1, interferon gamma, and TNF impair islet
function, decrease glucose levels and decrease glucose-
induced insulin release. Most of the deleterious effects
caused by cytokines on islet cells can be prevented by

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blocking NO production. Although some studies have shown
that isolated human islets are more resistant to the
suppressive effects of cytokines and NO than isolated
rodent islets, cytokines can suppress human islet
5   function irrespective of their effects on NO generation.

Cytokines and NO also influence the degree of
injury resulting from glomerulonephritis. TNF and IL-1
can increase the severity of glomerular injury in
nephritis and, therefore, may be important in modulating
10 glomerular injury clinically. Karker et al., _Clin. Expt._
Immunol. 90(2):312-318 (1992), have reported that agents
which suppress cytokines are effective in reducing
elevated albumin levels and tissue damage in
glomerulonephritis. Other research has demonstrated that
15 *in vivo* induction of inducible nitric oxide synthase
(iNOS) occurs in immune complex glomerulonephritis
(Jansen et al., _Kid. Internat._ 45(4):1215-1219 (1994)).

Cytokine regulatory agents of the present
invention can be used for treating a subject having a
20 disease mediated by nitric oxide and cytokines, such as
diabetes and glomerulonephritis. As used herein, the
term "treating" includes a meaning that encompasses
reducing or alleviating one or more symptoms or
conditions associated with a particular disease state
25 mediated by NO and cytokines. For example, treating
diabetes can be manifested by reducing glucose levels of
a diabetic.

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Effect of a Cytokine Regulatory Agent on Body Weight

Cytokine regulatory agents are useful for decreasing the body weight of a subject. In particular, the agents are useful for decreasing the weight of an

5   obese subject. A link between obesity and non-insulin dependent diabetes mellitus (NIDDM) is well known in the art (see Hotamisligil and Spiegelman, _Diabetes_ 43:1271-1278 (1994a)). Increased TNF-α expression has been detected in the adipose tissue of obese individuals and

10 has been suggested as a having a role in the appearance of NIDDM in these individuals (Hotamisligil et al., _J. Clin. Invest._ 95:2409-2415 (1995)). However, efforts to neutralize TNF activity using an antibody that binds the TNF receptor did not result in significant weight loss

15 when examined in a rat obesity/diabetes model, the Zucker fa/fa rat model (Hotamisligil et al., _J. Clin Invest._ 94:1543-1549 (1994b)).

Remarkably, administration of a cytokine regulatory agent, EX-2, to normal or obese rats resulted

20 in a significant decrease in the rate of body weight gain or a significant decrease in body weight, depending on the dose administered (see Example XX). As used herein, the term "decrease in body weight" is used broadly to mean an actual decrease in body weight or a decrease in

25 the rate of body weight gain over time, as compared to the normal weight gain expected in the period of time. As shown in Example XX, EX-2 also produced a small, but significant, decrease in the body weight of normal (non-obese) rats. These results indicate that a cytokine

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regulatory agent can cause a decrease in the rate of body weight gain and a decrease in body weight.

The weight loss observed in EX-2 treated rats correlated to an increased metabolic rate as determined

5   by increased resting oxygen consumption. These result demonstrate that a cytokine regulatory agent also is useful for increasing the resting oxygen uptake of a subject. As discussed above, a cytokine regulatory agent also can attenuate the decrease of maximal oxygen

10 consumption that occurs, for example, due to disuse deconditioning. The ability to increase maximal oxygen consumption in a subject can provide a significant clinical advantage to a subject suffering, for example, from congestive heart failure or other condition

15 characterized by a deficiency in oxygen uptake such as the decrease in oxygen consumption that occurs during deconditioning (see Example XIII). Furthermore, the disuse deconditioning results demonstrate that repeated treatment with a cytokine regulatory agent for at least

20 about two weeks can produce a positive adaptive change in a subject, resulting in increased aerobic capacity and exercise performance. Thus, a cytokine regulatory agent finds further use for increasing oxygen consumption in a subject. As used herein, the term "increasing oxygen

25 consumption" or "increasing oxygen uptake" is used broadly to mean an increase above a normal baseline level of resting or maximal oxygen uptake by a subject or a decrease in the expected loss of resting or maximal oxygen consumption.

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Treatment of Negative Side Effected Mediated
by Cancer Chemotherapy in Humans

Cytokine regulatory agents can be used to
ameliorate or decrease the negative side effects of
5   cancer therapeutic agents. A cytokine regulatory agent
can be co-administered with a cancer therapeutic agents
during a treatment regime for a cancer or can be co-
administered in a prophylactic regime, for example, with
Taxol°, which is administered to decrease the recurrence
10   of breast cancer.

Example XIX discloses the use of the cytokine
regulatory agent EX-2 to ameliorate the adverse side
effects associated with cancer chemotherapeutic agents
such as cisplatin, Taxol° and Adriamycin°, which induce
15   and enhance the production of cytokines.

EX-2 can be administered by intravenous
infusion to patients undergoing or about to undergo
cancer chemotherapy or cancer chemopreventive regimes.
For example, cisplatin, Taxol°, Adriamycin° or other
20   chemotherapeutic or radiologic treatment can be
co-administered with a cytokine regulatory agent in order
to reduce or inhibit the adverse side effects caused by
the chemotherapy or radiotherapy.

Increasing Levels of Cytokines in Mammals

25   A cytokine regulatory agent of the present
invention, or composition containing the agent, can also

be used to increase the physiologic level of one or more
cytokines. For example, a particular condition can
decrease the level or activity of a cytokine, which can
inhibit all or part of an immune response or the immune
5   system. Administration of a cytokine regulatory agent in
a pharmacologically efficacious dose can enhance the
level or activity of the cytokine, thereby permitting
reducing the level of immunosuppression.

        A cytokine regulatory agent can increase the
10 levels of IL-10 in a mammal such as a human (see Figures
    2 and 3). IL-10 can block the activation of some
    inflammatory cytokines, including TNF, IL-1 and IL-6,
    while up-regulating cytokines such as IL-12. IL-10 also
    stimulates the proliferation of mast cells and
15 thymocytes. IL-10 inhibits several monocyte and
    macrophage functions, including, for example, antigen
    presentation to T cells by depressing Class II MHC
    expression; synthesis of IL-1, IL-6, IL-8, CSF, and TNF;
    and microbicidal activities. The inhibited microbicidal
20 activities include suppressing production of nitrogen
    oxides and bactericidal metabolites. As a consequence of
    monocyte and macrophage IL-10 mediated inhibition,
    activity of some types of helper T cells is inhibited.
    Particularly, the T_H1 cells, which are responsible for
25 cell-mediated functions such as delayed-type
    hypersensitivity cells, and cytotoxic T cells are
    inhibited. As a further consequence T_H1 cell inhibition,
    activity of the T_H2 cells is augmented, particularly the T
    cell subset that augments B cell activation, bacterial
30 and helminthic resistance and allergic reactions. As

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disclosed herein, administration of a cytokine regulatory
agent can increase the plasma levels of IL-10 in mammals
(see Example XVII and XVIII and Figures 2 and 3) and,
therefore, can be useful for modulating, for example,
5   immunoresponsiveness in a subject.

Cytokine regulatory agents or pharmaceutical
compositions containing the peptides can be used for
treating any of the above-described pathologies and
conditions. One skilled in the art would know that a
10 pharmaceutical composition comprising a cytokine
regulatory agent can be administered to a subject having
elevated cytokine activity by various routes including,
for example, orally, intravaginally, rectally, or
parenterally, such as intravenously, intramuscularly,
15 subcutaneously, intraorbitally, intracapsularly,
intraperitoneally, intracisternally or by passive or
facilitated absorption through the skin using, for
example, a skin patch or transdermal iontophoresis.
Furthermore, the composition can be administered by
20 injection, intubation, orally or topically, the latter of
which can be passive, for example, by direct application
of an ointment or powder, or active, for example, using a
nasal spray or inhalant. A cytokine regulatory agent can
also be administered as a topical spray, in which case
25 one component of the composition is an appropriate
propellant. The pharmaceutical composition also can be
incorporated, if desired, into liposomes, microspheres or
other polymer matrices (Gregoriadis, Liposome Technology,
Vol. 1 (CRC Press, Boca Raton, FL 1984), which is
30 incorporated herein by reference). Liposomes, for

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example, which consist of phospholipids or other lipids, are nontoxic, physiologically acceptable and metabolizable carriers that are relatively simple to make and administer.

5   Cytokines can be expressed locally, such as at a site of localized infection, or can be expressed systemically, for example, as in disuse deconditioning or in an immune response. Cytokine expression can induce pyrexia (fever) and hyperalgesia (extreme sensitivity to 10   pain) in a subject, as well as macrophage and monocyte activation, which produces or further contributes to an inflammatory response in a subject. Since cytokine expression can be localized or systemic, one skilled in the art would select a particular route and method of 15   administration of the cytokine regulatory agent based on the source and distribution of cytokines in a subject. For example, in a subject suffering from a systemic condition, such as disuse deconditioning, a pharmaceutical composition comprising a cytokine 20   regulatory agent can be administered intravenously, orally or by another method that distributes the cytokine regulatory agent systemically. However, in a subject suffering from a pathology caused by localized cytokine expression, such as acute respiratory distress syndrome, 25   a cytokine regulatory agent can be suspended or dissolved in the appropriate pharmaceutically acceptable carrier and administered directly into the lungs using, for example, a nasal spray.

33

In order to regulate the biological activity of a cytokine, the cytokine regulatory agent must be administered in an effective dose, which is about 0.0001 to 0.5 mg/kg body weight per injection, or by

5   alternative modes of administration an effective dose is about 0.0001 to 100 mg/kg body weight. The total effective dose can be administered to a subject as a single dose, either as a bolus or by infusion over a relatively short period of time, or can be administered

10   using a fractionated treatment protocol, in which the multiple doses are administered over a more prolonged period of time. One skilled in the art would know that the concentration of a cytokine regulatory agent required to obtain an effective dose in a subject depends on many

15   factors including the age and general health of the subject as well as the route of administration and the number of treatments to be administered. In view of these factors, the skilled artisan would adjust the particular dose so as to obtain an effective dose for

20   efficaciously regulating cytokine activity.

Examples of cytokine regulatory agents and the effectiveness of a cytokine regulatory agent in preventing or minimizing or eliminating adverse biological effects mediated by cytokines are provided

25   below and summarized in Tables I-V. As described below, cytokine regulatory agents such as the peptides described in Example II can effectively regulate cytokine expression in mice (Examples III and IV) and provide relief from cytokine-mediated pain, swelling, fever and

30   lethality as demonstrated using mouse, rat and rabbit

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model systems that are recognized in the art as predictors of efficacy in humans (Examples V to XII).

The compounds described herein can be used as medicaments for the treatment of conditions such as
5   inflammation, pain, cachexia and patho-immunogenic diseases such as arthritis, inflammatory bowel disease, systemic lupus erythematosus and other autoimmune dysfunctions, which are characterized by altered cytokine activity. In addition, the Examples provide evidence
10 that a cytokine regulatory agent of the present invention is useful in attenuating the negative aspects of disuse deconditioning (Example XIII), protecting organs, particularly after ischemia-reperfusion injury and cyclosporine-induced nephrotoxicity (Example XIV),
15 reducing the nephrotoxic effects of cancer chemotherapy (Example XV), treating disease states associated with nitric oxide, including diabetes and glomerulonephritis (Example XVI) and decreasing the body weight of an individual (Example XX).

20   Yet further evidence that the agents of the present invention regulate cytokine expression and, therefore, are useful in treating the above-mentioned conditions, is provided in Examples XVII to XVIX. Increasing cytokine levels in mammals, including mice
25 (Example XVII) and humans (Example XVIII) subsequent to administration of a cytokine regulating agent provides additional evidence that the agents of the present invention regulate cytokine expression and activity.

35

        The following examples are intended to
illustrate, but not limit, the present invention.

                        EXAMPLE I

                 Synthesis of Peptide Cytokine
 5   Regulatory Agents

        This example describes methods for the solid
phase synthesis of peptide cytokine regulatory agents.

A.   Nle - Gln - His - (D)Phe - Arg - (D)Trp - Gly-NH$_2$

        A peptide cytokine regulatory agent having the
10   amino acid sequence, Nle-Gln-His-(D)Phe-Arg-(D)Trp-Gly
     ("EX-1"), was synthesized using a modification of the
     solid phase peptide synthesis method of Merrifield
     (1964). Essentially, MBHA resin containing a t-BOC
     glycine derivative (Advanced Chemtech; Louisville, KY)
15   was added to a reaction vessel suitable for solid phase
     peptide synthesis (see Houghten (1985), *supra*). The
     resin was washed three times with methylene chloride and
     the t-BOC protecting group was removed using
     trifluoroacetic acid (TFA) containing 1-2% anisole in
20   methylene chloride. The resin then was washed with
     methylene chloride and treated with disopropylethylamine.

        The peptide was extended by the addition of 3.2
     equivalents of N-formyl-BOC-protected D-tryptophan in
     dimethylformamide and 3.0 equivalents of
25   dicyclohexylcarbodiimide. The reaction was monitored
     using ninhydrin and was allowed to proceed for 25 min,

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after which the resin was washed using methylene
chloride. The procedure was repeated using di-tolulyl-
BOC arginine, then with each of the desired protected
amino acids until the complete heptapeptide was
5 synthesized.

        Following synthesis of the heptapeptide, the N-
formyl protecting group on the tryptophan residue was
removed using 20% piperidine in DMF and the resin was
washed with methylene chloride. The peptide was cleaved
10 from the resin using anhydrous hydrogen fluoride (HF)
containing 10% anisole, the reaction mixture was
concentrated and the residue was extracted with aqueous
acetic acid. The acetic acid fraction, which contained
the dissolved sample, was removed and the residue was
15 washed with water. The wash was added to the acetic acid
fraction and the combined sample was concentrated. The
resulting crude peptide was purified by RP-HPLC (Vydac,
C-18 column, using a gradient of 1 to 60% solution B over
30 min (solution A is 0.1% TFA/water and solution B is
20 0.1% TFA/acetonitrile).

        The peptide was determined to be 97% pure by
RP-HPLC (Vydac C-18 column, using isocratic 24% solution
B; solution A and solution B, as above; absorption
determined at 215 nm). The mass of the purified
25 heptapeptide was determined by plasma desorption mass
spectrometry using a BioIon 20 Mass Analyzer time of
flight detector. The mass of the EX-1 peptide was
measured to be 942.7, which was essentially the same as
the expected molecular mass (MS (M+1) = 942.2).

B. His-(D)Phe-Arg-{(D)Trp(CH₂)}-(NAc)Gly-NH₂

A cytokine regulatory agent of the invention,
having the amino acid sequence His-(D)Phe-Arg-
{(D)Trp(CH₂)}-(NAc)Gly-NH₂, was synthesized and purified
5   as described above, except for the following
modifications. Boc-(D)Trp was converted to the
corresponding N,O-dimethylhydroxamate using methyl
chloroformate and N,O-dimethylhydroxyl amine
hydrochloride. Reduction of the tryptophan amide with
10   lithium aluminum hydride gave the Boc-(D)Trp aldehyde.

A solution of the Boc-(D)Trp aldehyde and
sodium cyanoborohydride in DMF was added to glycine
attached to the Rink amide resin in DMF containing 1%
acetic acid. After the reductive amination was complete
15   the secondary amine was acetylated with acetic anhydride
and the resin was shaken with 1:1 trifluoroacetic acid
and methylene chloride to remove the Boc group.
Sequential coupling of the remaining amino acids was
performed on an peptide synthesizer (Applied Biosystems)
20   to produce the peptide His-(D)Phe-Arg-{ (D)Trp(CH₂) }-
(NAc)Gly-NH₂. The peptide was cleaved from the resin and
purified as described above.

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EXAMPLE II

Preparation of Acetylated Peptide
Cytokine Regulatory Agents

This example describes methods for preparing
5   N-acetylated peptide cytokine regulatory agents.

The heptapeptide Nle-Gln-His-(D)Phe-Arg-
(D)Trp-Gly-NH$_2$ was synthesized as described in Example
I.A., except that prior to cleaving the newly synthesized
peptide from the resin, the amino terminus of the peptide
10 was acetylated by treating the sample with acetic
anhydride, diisopropylethylamine and methylene chloride
for 2 hr. Following acetylation, the heptapeptide was
cleaved from the resin, purified by RP-HPLC and
characterized by mass spectrometry, as described above.
15 The acetylated heptapeptide of Example II, designated,
here, as EX-2, was determined to be 98% pure and the mass
was measured to be 985.2 daltons, which was same as the
expected molecular mass.

Similar methods as described in Examples I and
20 II were used to synthesize other cytokine regulatory
agents of the invention, including Ac-(cyclohexyl)Gly-
Gln-His-(D)Phe-Arg-(D)Trp-Gly-NH$_2$ ("EX-3"); Ac-(D)Phe-Arg-
(D)Trp-NH$_2$ ("EX-4"); Ac-His-(D)Phe-Arg-(D)Trp-Gly-NH$_2$
("EX-5"); and Ac-His-(D)Phe-Arg-(D)Trp-NH$_2$ ("EX-6"). Ac-
25 His-(D)Phe-Arg-{ (D)Trp(CH$_2$) }-(NAc)Gly-NH$_2$ was prepared
using the method described in Example I.B. except that,

prior to cleaving the peptide from the resin, the peptide was acetylated using excess acetic anhydride.

EXAMPLE III

Reduction of Lipopolysaccharide-Induced
5   Tumor Necrosis Factor Levels in Mice

          This example describes the effectiveness of two cytokine regulatory agents for decreasing tumor necrosis factor (TNF) levels in lipopolysaccharide (LPS; endotoxin) treated mice.

10   Balb/c female mice weighing approximately 20 g were placed into two groups, a control group and a treated group. Five mg/kg of LPS in 0.9% saline was administered by intraperitoneal (IP) injection into the control mice. Mice in the treated group were first
15   injected IP with 30 μg EX-2 or 150 μg EX-3 in saline, then, one min after EX-2 or EX-3 was administered, the mice received LPS as described for the control group.

          Blood samples were collected from the orbital sinus of treated and control mice at various times up to
20   four hr after LPS was administered. The plasma was separated by centrifugation at 3000 x g for 5 min, then diluted with four volumes of 1x phosphate buffer saline (pH 7.4) containing 1% bovine serum albumin. A 100 μl sample of serum was assayed by ELISA for TNF-α (Genzyme;
25   Cambridge MA).

40

The mean (+/- SEM) TNF-α level in six mice from each group was determined and the percent reduction in TNF levels was calculated. As shown in Table I, treatment of mice with EX-2 resulted in a 50% decrease in

5   the level of TNF-α as compared to untreated control mice. Similarly, treatment of mice with EX-3 resulted in a 56% decrease in the level of TNF-α as compared to untreated control mice and treatment with EX-4 resulted in a 53% decrease (Table II). These results indicate that the

10 peptides of the invention can restrain LPS-induced cytokine activity.

TABLE I

BIOLOGICAL DATA FOR CYTOKINE REGULATORY AGENT, EX-2

Biological Test	Dose	Efficacy
Reduction in TNF levels	30 μg/mouse	50%
Reduction in IL-6 levels	300 μg/mouse	60%
Reduction in Carageenan-induced Paw Swelling	1 μg/mouse	45%
Inhibition of LPS-induced Lethality	11 x 300 μg/mouse	83%
Reduction in IL-1-induced Hyperalgesia	1 μg/mouse	125%
Reduction in LPS-induced PMN Count	100 μg/kg	58%
Reduction in IL-1-induced Fever	500 μg/kg	52%
Reduction in LPS-induced Fever	50 μg/kg 150 μg/kg	45% 52%
Reduction in arachidonic acid-induced Ear Swelling	100 μg/mouse	72%
Reduction in Morphine-induced Respiratory Depression	10 + 20 + 20 μg/kg/rabbit	50%

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TABLE II

BIOLOGICAL DATA FOR CYTOKINE REGULATORY AGENTS, EX-3 AND EX-4

Biological Test	Dose	Efficacy	
		EX-3	EX-4
Reduction in TNF levels	150 μg/mouse	56%	53%
Reduction in Carageenan-induced Paw Swelling	1 μg/mouse	49%	NT
Inhibition of LPS-induced Lethality	11 x 300 μg/mouse	86%	75%
Reduction in LPS-induced Fever	150 μg/kg	57%	52%
Reduction in arachidonic acid-induced Ear Swelling	100 μg/mouse	62%	NT
Reduction in Morphine-induced Respiratory Depression	10 + 20 + 20 μg/kg/rabbit	65%	NT

NT = not tested

EXAMPLE IV

Reduction of Lipopolysaccharide-Induced Interleukin-6 Levels in Mice

This example describes the effectiveness of a cytokine regulatory agent for decreasing Interleukin-6 (IL-6) levels in LPS treated mice.

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Balb/c mice were grouped and treated as described in Example III, above. Blood samples were obtained from the orbital sinus at various times up to six hr and serum was collected and diluted as described

5   above. A 100 μl aliquot was assayed for IL-6 levels using an IL-6-specific ELISA by a modification of the method of Starnes et al., J. Immunol. 145:4185-4194 (1990), which is incorporated herein by reference.

The mean (+/- SEM) IL-6 level in six mice from

10 each group was determined and the percent reduction in IL-6 was calculated. As shown in Table I, treatment of mice with EX-2 resulted in a 60% decrease in the level of IL-6 as compared to untreated control mice.

EXAMPLE V

15   Carageenan-Induced Paw Swelling

This example describes the effectiveness of two cytokine regulatory agents for alleviating inflammation and pain.

Carageenan-induced paw swelling was induced

20 using a modification of the methods of Hiltz and Lipton, Peptides 11:979-982 (1990); Vinegar et al., Fed. Proc. 46:118-126 (1987); and Vinegar et al., J. Pharmacol. Expt. Therap. 166:96-103 (1969), each of which is incorporated herein by reference. Briefly, adult female

25 Balb/c mice were anesthetized by IP injection of 7 mg/kg ketoamine and 0.6 mg/kg rompun. Foot pad thickness was measured using a spring loaded micrometer (Swiss

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Precision Instruments). Foot pad thickness was expressed
in units of 1/100 inch. After baseline measurements were
obtained, mice were injected into a hind foot pad with
either 0.2 ml physiologic saline (control) or varying

5   doses of EX-2 or EX-3 in 0.2 ml saline (treated). The
first injection was followed immediately by injection of
0.02 ml of 0.15% κ-carageenan (Sigma Chemical Co.; St.
Louis, MO.).

Hind foot pad thickness was measured hourly for

10 six hr, the change in thickness was determined and the
percent reduction in swelling due to treatment with EX-2
was calculated. As shown in Tables I and II, IP
injection of 1 μg EX-2 or 1 μg EX-3 reduced carageenan-
induced swelling by 45% or 49%, respectively, when

15 measured at the 2 hr time point.

EXAMPLE VI
Lipopolysaccharide-Induced Lethality

This example describes the effectiveness of the
cytokine regulatory agents, EX-2, and EX-3 and EX-4, in

20 reducing lethality from sepsis induced by administration
of LPS.

These experiments were performed based on
information reported by Rivier et al., Endocrinology
125:2800-2805 (1989), which is incorporated herein by

25 reference. Adult female Balb/c mice were provided food
and water ad libitum. Mice were injected IP every four
hr for 40 hr with 30 to 300 μg EX-2, EX-3 or EX-4 in 0.2

ml saline (treated group) or with 0.2 ml saline, alone
(control group) (10 mice per group). Immediately
following the first injection, 0.6 mg LPS endotoxin in
0.2 ml saline was administered to each mouse. Following

5   LPS injection, EX-2 or saline was administered to the
treated mice or the control mice, respectively, every 4
hr for 36 hr.

As shown in Tables I and II, administration of
3.3 mg EX-2, EX-3, or EX-4 (11 injections of 300 μg each)

10 produced an 83%, 86%, and 75%, respectively, increase in
survival as compared to control mice. These results
demonstrate that intraperitoneal administration of the
cytokine regulatory agents of the invention can reduce
lethality due to LPS-induced sepsis.

15   **EXAMPLE VII**
        Reduction in Interleukin-1ß-Induced Hyperalgesia

        This example describes the effectiveness of a
cytokine regulatory agent, EX-2, in providing pain
prophylaxis.

20   These experiments were performed using a
modification of the methods described by Poole et al.,
Br. J. Pharmacol. 106:489-492 (1992); Follenfant et al.,
Br. J. Pharmacol. 98:41-43 (1989); and Randall and
Sellito, Arch. Internatl. Pharmacodyn. 111:409-419

25 (1957), each of which is incorporated herein by
reference. Adult male Sprague-Dawley rats (175-275 g)
were tested for hyperalgesia by a paw pressure technique

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using variable pressure instrumentation (IITC Life
Sciences; Woodland Hills, CA). Rats were acclimated to
the housing environment and were handled for three days
prior to beginning a training session. On the day before

5   the hyperalgesia experiments was to begin, each rat was
placed into a sock and two variable paw pressure tests
were performed 15 min apart. The next day, the rats were
pretested to determine the pressure (mm Hg) at which each
animal exhibited escape reflexes such as whole body

10 struggling and/or vocalization. Approximately 5-10% of
the rats were non-responders and were eliminated from
further experiments.

Animals that responded to the paw pressure were
pretreated by IP injection of various concentrations of

15 EX-2 in a volume of 1 ml/kg (treated) or saline, alone
(control). After 20 min, 100 μl of IL-1ß (1U/100 μl) was
administered to rats via intra plantar injection. Two hr
after IL-1 administration, rats were subjected to two
additional paw pressure tests and the increase in mm Hg

20 of pressure that could be applied to the EX-2-treated
rats as compared to the control rats was determined. As
shown in Table I, treatment with 1 μg EX-2 increased the
amount of pressure the rats would tolerate by 125% as
compared to the control rats.

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EXAMPLE VIII

Adult Respiratory Distress Syndrome

This example describes the effectiveness of a
cytokine regulatory agent, EX-2, in minimizing
5   respiratory distress syndrome in LPS-treated rats.

These experiments were performed using a
modification of the methods described by Ulich et al.,
Am. J. Pathol. 141:61-68 (1992) and by Wheelden et al.,
Lab. Animals 26:29-37 (1992), each of which is
10 incorporated herein by reference. Male Harlan Sprague-
Dawley rats were anesthetized using a mixture of 70 mg/kg
ketamine and 6 mg/kg rompun injected IP. A 2-3 cm
incision was made in the neck of each anesthetized rat
and its trachea was exposed by blunt dissection of the
15 surrounding soft tissue. The rats were suspended on a
near vertical slab and intratracheal injections were
performed by inserting into the exposed trachea, at a
point 1 cm posterior to the larynx, a 25G x ½ inch needle
attached to a 1 cc syringe.

20   Each rat received 0.5 ml/kg of saline or 0.5
ml/kg of 10 mg/ml (5 mg/kg) LPS endotoxin via slow
intratracheal administration. Immediately following
administration of the LPS endotoxin, rats were injected
IP with 1 ml/kg of either saline (control) or saline
25 containing various concentrations of EX-2 (treated). The
rats were maintained in the elevated position for 1-2 min
to facilitate distribution of the LPS and saline into the
lung. The incisions were closed and the rats were

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allowed to recover. Two and four hr post-intratracheal
injection, saline or EX-2 again was administered IP to
control and treated rats, respectively.

       At 6 hr post-intratracheal injection, the rats
5   were re-anesthetized and exsanguinated via cardiac
puncture. Serum was collected and saved. The neck and
chest were opened to expose the trachea and lungs, the
lungs were lavaged with 6 x 5 ml saline using a 27G x 3/4
inch needle and the lavage fluid was pooled.

10   The total polymorphonuclear leukocytes (PMN;
neutrophils) in the broncho-alveolar lavage fluid were
counted in the EX-2-treated rats and compared with the
number in the control rats. As shown in Table I,
treatment with 100 μg/kg EX-2 inhibited the increase in
15   PMN infiltration in LPS-treated lungs by 58%.

EXAMPLE IX
Inhibition of Interleukin-1ß- or Lipopolysaccharide-Induced Temperature Increase

       This example describes the effectiveness of the
20   cytokine regulatory agents, EX-2, EX-3 and EX-4, at
inhibiting body temperature increase in rats in response
to two different agents.

       Male Wistar rats (45-75 days old) were placed
in a temperature controlled room held at 26°C, which is
25   thermoneutral for the normal body temperature of rats,
and were maintained in the room with free access to food

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and water for 24 hr prior to testing. On the morning of
the study, rats were marked for identification and
weighed. The temperature of each rat was determined by
placing the animal in a regulatory cage designed to
5   minimize stress and inserting a temperature probe (YSI
probe # 402) 3-5 cm into the animal's rectum. The
temperature was recorded 15 sec after the reading
stabilized. Measurements were repeated 1 hr later to
establish a baseline temperature for each rat.

10   After the baseline temperatures were
established, rats were injected IP with saline, IL-1ß or
LPS endotoxin. Rats then were injected IP with either
saline (control) or various concentrations of EX-2, EX-3,
or EX-4 (treated). The temperature of the rats was
15   measured every hr for 6 hr and the inhibition by EX-2,
EX-3, or EX-4 of the rise in temperature due to IL-1ß or
LPS was determined.

              As shown in Table I, treatment with 500 μg/kg
EX-2 inhibited IL-1-induced fever by 52%. In addition,
20   treatment with 50 or 150 μg/kg EX-2 inhibited LPS-induced
fever by 45% or 52%, respectively, when measured 6 hr
following LPS injection. Furthermore, treatment with 150
μg/kg EX-3 or EX-4 inhibited LPS-induced fever by 57% and
52%, respectively (Table II). These results demonstrate
25   that various cytokine regulatory agents of the invention
can effectively reduce fever.

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EXAMPLE X

Reduction of Arachidonic Acid-Induced
Ear Swelling in Mice

This example demonstrates that EX-2 and EX-3

5   can reduce arachidonic acid-induced ear swelling in mice.

Experiments were performed using female Balb/c

mice weighing 18-23 grams.   Saline or 100 μg EX-2 or EX-3

was administered IP, 30 min prior to topical application

of arachidonic acid (AA).   A 10 μl pipet was used to

10   apply 10 μl AA solution (100 mg/ml ethanol; Calbiochem-

Novabiochem; San Diego CA) to the inner and outer

surfaces of the right ear of each mouse.   Ten μl ethanol,

alone, was applied to the inner and outer surface of the

left ear of each mouse.

15   Ear thickness was measured with a hand-held

spring loaded caliper immediately before and 60 min after

AA application.   Increase in ear thickness was calculated

by subtracting the change observed in the control ear

from the change observed in AA-treated ear.   The value

20   for each group (saline and control) is the average of the

swelling observed in the individual mice in each group.

The percent reduction of swelling is based on the

swelling observed in the saline control group.   As shown

in Tables I and II, EX-2 and EX-3 reduced AA-induced ear

25   swelling by 72% and 62%, respectively.

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EXAMPLE XI

Reduction of Morphine-Induced Respiration Depression in Rabbits

This example demonstrates that EX-2 and EX-3
can reduce the depression in respiration induced by
morphine in rabbits.

Male Shelton rabbits (3-4 kg) were restrained
and fitted around the thorax, just behind the front
limbs, with a respiration transducer (Model F-RCT; Grass
Instruments; Quincy MA). The transducer was connected to
a grass polygraph via an EKG cable. An intravenous line
was established for drug administration by cannulating
the marginal ear vein using a 25G butterfly needle.

Rabbit breathing was allowed to stabilize, then
morphine sulfate (2 mg/kg in 0.5 ml saline) was
administered by intravenous (iv) injection and
respiratory rate and depth were monitored for 10 min. A
second dose of morphine was administered, then, after 10
min, EX-2 or EX-3 (10 µg/kg in 0.5 ml saline) was
administered, iv, and rabbits were monitored for 20 min.
Two additional doses of EX-2 or EX-3 (20 µg/kg in 1.0 ml
saline) were administered at 20 min intervals, i.e., 40
min and 60 min after the first morphine injection.

Results were calculated as the percent change
from baseline values and are expressed as the difference
of the mean value of the treated group minus the mean
value of the control group at the end of the experiment

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(80 min). As shown in Tables I and II, EX-2 and EX-3
reduced the morphine-induced respiratory depression in
rabbits by 50% and 65%, respectively.

EXAMPLE XII

5   Effect of Orally Administered Cytokine Regulatory
            Agents in Reducing TNF-α Levels and
                    LPS-induced Lethality

        This example describes the oral effectiveness
of various cytokine regulatory agents in reducing LPS-
10   induced TNF-α levels and lethality in mice.

        The LPS-induced lethality studies were
performed based on information reported by Rivier et al.,
supra, 1989. Adult female Balb/c mice were provided food
and water ad libitum. Mice were administered 150 μg or
15   300 μg EX-2, EX-3, EX-5 or EX-6 in 100 μl saline by
gavage every 4 hr for 40 hr (total doses of 1.65 mg and
3.3 mg, respectively). Control mice received 100 μl
saline, alone. Immediately following the first dose of
cytokine regulatory agent or saline, 0.6 mg LPS in 0.2 ml
20   saline was administered by IP injection. A statistically
significant increase in survival was observed in mice
receiving 3.3 mg EX-5 (63%), 1.65 mg EX-6 (68%) or 3.3 mg
EX-5 (44%) as compared to control mice (0%) or mice
receiving EX-2 or EX-3 (0% to 11%).

25   The ability of orally administered cytokine
regulatory agents to reduce LPS-induced TNF-α levels also
was examined. Balb/c female mice (20 g) were
administered 150 μg or 300 μg EX-2, EX-3, EX-5 or EX-6 in

100 μl saline by gavage. Control mice received 100 μl
saline, alone. One min later, 0.1 mg LPS was
administered by IP injection. Samples were collected and
TNF-α levels were determined as described in Example III,
5   above.

The mean TNF-α levels in the mice from each
group (n = 9-20) was determined and the percent reduction
in TNF-α levels was calculated. TNF-α levels were
significantly reduced in mice receiving 150 μg EX-3
10 (49%); 300 μg EX-3 (40%) or 300 μg EX-5 (44%) as compared
to control mice (0%) and mice receiving EX-2 (26% to
28%). These results demonstrate that various cytokine
regulatory agents of the invention are effective when
administered orally.

15   **EXAMPLE XIII**

                      Disuse Deconditioning in Rats

        This experiment describes the effectiveness of
a cytokine regulatory agent, EX-2, in ameliorating the
negative effects associated with 14 days disuse
20 deconditioning in rats.

        Adult male Sprague-Dawley rats (355-365 g) were
used in this study. Animals were housed individually in
standard rat cages and were acclimated on a treadmill for
4 days. The next day, a baseline tail bleed was
25 performed, followed by a maximal treadmill stress test
the day after. The day following the stress test, the
animals were divided into the appropriate treatment and

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control groups and the animals were suspended by their
tails in suspension cages, which simulates weightlessness
and, therefore, deconditions the animals. Another group
of control animals was placed in standard rat cages with

5 grated flooring similar to that utilized in the
suspension cages. All animals were housed one per cage
and supplied with food and water *ad libitum*. Body
weights and food consumptions were measured daily.
Animals received twice daily IP injections of 1 μg/kg or

10 5 μg/kg body weight of EX-2 (treated), or 1 ml/kg body
weight of saline (control). On day 10, all animals were
stress tested on the treadmill until they reached
exhaustion. Data on maximum VO_2 and running time were
collected and compared to pre-suspension results. On day

15 14, all animals were sacrificed, and blood, tissues, and
organs were collected for further analysis. All
procedures and tests were performed blind to avoid
researcher subjectivity. Statistical significance
($p < 0.05$) between groups was evaluated by ANOVA with

20 Student-Newman-Keuls test for multiple comparisons. All
data are reported as the mean $\pm$ standard error (SE).

Body weights decreased significantly for all
suspended animals, regardless of treatment. Food
consumption was not significantly different between the

25 groups. All of the animals were subjected to the stress
tests, which measured exercise time and maximal VO_2. As
well, levels of the oxidative enzyme soleus citrate
synthase and tibia bone density were measured.

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As summarized in Table III, EX-2 exhibits a
significant effect on many of the parameters associated
with disuse deconditioning. Twice daily injections of
low doses of EX-2 (1 or 5 μg/kg) significantly attenuated
5   the deconditioning effects on exercise time, maximal VO_2,
soleus citrate synthase levels, and tibia bone density.
More specifically, EX-2 significantly attenuated both
exercise performance parameters, exercise time and
maximal VO_2, 44% and 80%, respectively, when compared to
10   the decrease between the saline control and suspended
animals. Soleus citrate synthase was attenuated by 50%.
Tibia bone density measurements showed that EX-2
significantly attenuated suspension-induced bone
demineralization by 50%. These results demonstrate that
15   administration of a cytokine regulatory agent of the
present invention reduces the negative effects of disuse
deconditioning.

TABLE III

DISUSE DECONDITIONING

Variable	% Attenuation *
Exercise Time	44% **
Maximal VO$_2$	80% **
Soleus Citrate Synthase	50% **
Tibia Bone Density	50% **

* - % attenuation of suspension-induced decrease of
       suspended groups as compared to control group.
** - significantly greater than suspended controls
       ($p < 0.05$).

EXAMPLE XIV

Organ Protection

This example describes the effectiveness of a
cytokine regulatory agent, EX-2, in preventing kidney
damage in two rat models, an ischemia-reperfusion model
and a cyclosporine-induced nephrotoxicity model.

A. Ischemia-Reperfusion Injury:

These experiments were performed to determine
if a peptide which has cytokine regulatory properties,
EX-2, can ameliorate the reperfusion-induced
vasoconstriction and reduction in glomerular filtration

rate that results from clamping of the renal artery. As a comparison, the NSAID ketorolac was utilized in the same model.

Sprague-Dawley rats (250-300 g) were
5   anesthetized with Inactin* (120 mg/kg). After deep anesthesia was assured, the rats were cannulated in the trachea with polyethylene (PE) tubing (PE 240), jugular vein, femoral artery, bladder (PE 50 for previous 3 cannulations), and left ureter (stretched PE 50). Once
10   cannulation was completed, a 2 cm flank incision above the left kidney was performed and the renal pelvis and vascular bundle were exposed. The tissue surrounding the left renal artery was gently dissected away from the artery. The exposed renal artery was clamped for 1 hr
15   using a small vascular cross-over clamp.

Just prior to clamping, EX-2 (50 or 500 μg/kg), ketorolac (KETO, 1 mg/kg), or vehicle was administered intravenously (iv) After 1 hr of clamping (ischemia), the clamp was gently removed with care taken to avoid
20   damaging either the renal artery or nerves. An infusion of ^{3}H-Inulin (NEN/Dupont; Wilmington, DE), (0.6 μCi/ml in phosphate buffered saline (PBS)) (2 ml/hr) was initiated at a constant rate of 4 μCi/hr. After 60 min of reperfusion, glomerular filtration rate (GFR), renal
25   plasma flow (RPF) and renal vascular resistance (RVR) were assessed in two 30 min collection periods. The kidney was then removed for analysis. Sham operated and clamped rats served as controls for the above group. Contralateral renal function was utilized to determine

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the stability of the rat's renal function during the
measured period. The results demonstrate that EX-2
effects an increase in glomerular filtration rate and
decrease in vascular resistance (Table IV). This data
5 provides that a present cytokine regulatory agent
effectively protects the kidney against damage associated
with ischemia-reperfusion.

TABLE IV

10   **RENAL ISCHEMIA/REPERFUSION (I/R)**

	GFR	RVR (ml/mm)	RPF
Sham	1.0±0.1	1.0±0.1	4.2±0.4
I/R + PBS	0.2±0.1	6.6±2.1	0.8±0.2
I/R + EX-2	0.5±0.1	1.8±0.2	2.3±0.3

B. Cyclosporine-Induced Nephrotoxicity:

        This experiment was performed to ascertain if
20 EX-2 can mitigate the reduction in glomerular filtration
rate (GFR) and renal blood flow, as well as the
dysfunction in fluid and electrolyte homeostasis induced
by immunosuppressive treatment with cyclosporine A.

In the first study, male Sprague-Dawley rats
(n=43) with a weight range of 240-290 g at the initiation
of the cyclosporine A (CsA) treatment were divided into 4
weight matched groups. The groups were as follows:

5   (1) Control: no CsA, no EX-2, treatment only with CsA
vehicle (PBS) PO and EX-2 vehicle (also PBS) IP; (2) CsA:
treatment with CsA (50 mg/kg in PBS administered PO daily
at 0830 hr for 9 days) and IP injections of PBS (Ex-2
vehicle); (3) CsA and 50 μg: CsA treatment identical to

10 group 2 with the addition of EX-2 (50 μg/kg IP
administered daily at 0800 and 1700 hr in EX-2 vehicle);
and (4) CsA and 500 μg: CsA treatment identical to groups
2 and 3, and EX-2 treatment protocol similar to group 3
except that the dose of EX-2 was 500 μg/kg BW. After

15 deep anesthesia was assured, the rats were cannulated in
the trachea (PE 240) jugular vein, femoral artery, and
bladder (PE 50 for the previous 3 cannulations). After
cannulation, infusion of ^{3}H-inulin was initiated 50 min
prior to the measurement period to assess glomerular

20 filtration rate, renal blood flow and plasma flow. Renal
blood flow was determined by the inulin extraction
technique. At the end of the study, the kidneys were
removed for histological analysis.

In the second study, male Sprague-Dawley rats
25 (n=31) with a weight range of 240-290 g at the time of
initiation of the cyclosporine treatment were divided
into 4 weight-matched groups. The time course and
protocol of this study matched the first study with the
following exceptions: (1) The daily dose of CsA was
30 reduced to 30 mg/kg and was administered subcutaneously

(SC) and (2) the 50 μ/kg dose of EX-2 was substituted
with a twice daily dose of 200 μg/kg. The 500 μg/kg dose
of EX-2 was repeated in this study. Renal hemodynamics
were determined and extracellular fluid volumes were also
5   assessed.

         CsA treatment did not significantly decrease
renal blood flow in these studies due to the scatter in
the data. However, EX-2 treatment improved blood flow to
values not different from control. In the second study,
10   where extracellular fluid volumes (ECF) were measured,
there were no differences in ECF among control and
treatment groups (27.6 $\pm$ 1.9 in control, 27.5 $\pm$ 1.7 in
CsA and vehicle, 26.2 $\pm$ 0.9 in CsA + 200 μg/kg EX-2, and
25.7 $\pm$ 1.4% in CsA + 500 μg/kg EX-2).

15   Urinary protein excretion was also measured and
the data demonstrated that EX-2 treatment did not
increase urinary protein excretion compared to CsA-
treated rats despite the significant increase in GFR.
EX-2 significantly increased GFR, compared to CsA and
20   vehicle controls, in both studies and returned renal
blood flow to values not different from control. There
were no changes in ECF as a result of EX-2 treatment and
there was a trend to decreased urinary protein excretion
normalized to glomerular filtration rate. The results
25   from the two experiments are shown in Figures 1a and 1b
and provide evidence that a cytokine regulatory agent of
the present invention reduces cyclosporine-induced
nephrotoxicity and the damage caused thereby.

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EXAMPLE XV

Cancer Chemotherapy

This example describes the effectiveness of a
cytokine regulatory agent, EX-2, in reducing the
5   nephrotoxic effects of cancer chemotherapeutic agents.

Male Sprague-Dawley were used to determine the
effect of EX-2 on renal function after cisplatin (CDDP)
administration. All rats were administered CDDP (4 mg/kg
IP) and divided in 3 treatment groups (n=6/group). The
10   groups were as follows: (1) Administration with vehicle
and sterile PBS 3x/day; (2) Administration of EX-2 at a
dose of 5 μg/kg, 3x/day; and (3) same as group 2 except
the dose of EX-2 was 50 μg/kg, 3x/day.

An infusion of ^{3}H-inulin (0.6 μCi/ml in PBS) (2
15   ml/hr) was initiated at the end of the surgical
preparation period. The continuous infusion was started
60 min prior to the renal function measurements and
maintained to the end of the study. Glomerular
filtration was assessed by inulin clearance techniques in
20   two 30 min periods and renal blood flow (RBF) and renal
plasma flow (RPF) were measured by inulin extraction
techniques (arterial vs. renal vein inulin concentration)
from left kidney renal vein blood sampled at the end of
the second GFR measurement period. Urinary output was
25   also monitored to determine both urine flow and
fractional excretion of water. Urinary electrolyte (Na$^.$
and K$^.$) concentrations were analyzed by flame photometry
(Model 51 Ca, Bacharch, Inc.; Pittsburgh, PA) and urinary

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protein concentrations were determined. At the end of

the measurement periods, the kidneys were removed for

histological analysis. The influence of varying

treatment doses of EX-2 on renal function in 3 CDDP

5 administered rats (CDDP at 4 mg/kg IP) is summarized in

Table V. The data demonstrates that a cytokine

regulatory agent of the present invention increases

glomerular filtration rate, renal plasma flow and renal

blood flow, thereby reducing the nephrotoxicity of a

10 cancer chemotherapeutic agent.

TABLE V

		CANCER CHEMOTHERAPY	
	GFR	RPF	RBF
		(ml/mm)	
CDDP + vehicle	1.0 ± 0.4	5.6 ± 0.9	10.2 ± 1.6
CDDP + 5 μg/kg	0.8 ± 0.2	6.5 ± 1.0	12.1 ± 2.0
CDDP + 50 μg/kg	1.6 ± 0.4	8.8 ± 1.1	16.8 ± 2.2

Example XVI

Treatment of Diseases Mediated by

Nitric Oxide and Cytokines

This example describes the effectiveness of

25 EX-2 at inhibiting inducible nitric oxide synthase (iNOS)

and decreasing glucose in streptozotozin-induced diabetes

and in reducing albumin levels in glomerulonephritis.

A.   Inducible Nitric Oxide Synthase (iNOS):

Rat aorta smooth muscle cells (RASMC) were
isolated from male rats (325-350 g) using the previously
published procedures of Gelsterfer et al., _Circ. Res._
5   62:749-756 (1988), which is incorporated herein by
reference. Cells were positively identified as smooth
muscle cells by indirect immunofluorescent staining for
anti-α-actin, using a mouse anti-α-actin antibody and
anti-mouse IgG FITC conjugate. Cells were grown in T-75
10   tissue culture flasks (Corning Delbelco's Glass, Inc.;
Corning, NY) in 50% F12 and 50% Delbelco's Modified Eagle
Medium (DMEM) supplemented with 10% bovine serum, 0.2 g/1
L-glutamine, penicillin (100 U/ml) and streptomycin (0.1
mg/ml).

15   Cells between passages 1-5 were pretreated with
one of the following: vehicle, LPS (1 μg/ml), IL-1β (10
U/ml), or TNF-α (100 U/ml) for 6 hr. In similar groups,
0.1 or 1 μM of EX-2 was added 10 min before the addition
of LPS, IL-1β or TNF-α. Six hr later, cells were washed
20   with Earle's balanced salt solution and incubated for 30
min with vehicle of 100 μM L-nitro arginine methyl ester
(L-NAME). Accumulation of cGMP was determined following
exposure to 0.3 mM 3-isobutyl-1-methyl xanthine (IBMX) in
the presence or absence of 1 mM L-arginine. Medium was
25   then rapidly aspirated and 500 μl of 0.1 N HCl was added
to each well to stop the reactions and extract cGMP.
Thirty min later, the HCl extract was collected and cell
remnants removed from the wells by adding hot 1.0 N NaOH
and scraping the well with a rubber policeman. The HCl

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extract was analyzed for cGMP by RIA and the NaOH-
solubilized cell remnants were used for protein
determination.

Male Sprague-Dawley rats were anesthetized with
5   55 mg/kg pentobarbital IP. Catheters were inserted into
the carotid artery and jugular vein for blood withdrawal
and drug administration, respectively. EX-2 or saline
was given to a group of 6 rats. One hr later, while rats
were still under anesthesia, blood was withdrawn into a
10 heparinized syringe and plasma was obtained by
centrifugation. RASMC were incubated with plasma from
control or EX-2-treated animals and the ability of plasma
components to inhibit the induction of INOS by LPS or IL-
1β was determined as above.

15   The radioligand (^{125}I-succinyl cGMP-tyrosine
methyl ester) was prepared by the method of Hunter and
Greenwood Nature, 94:495-496 (1962), which is
incorporated herein by reference, using carrier free ^{125}I.
The iodination reaction products were separated by RP-
20 HPLC following the procedures of Patel and Lindent, Anal.
Biochem. 168:417-425 (1988), which is incorporated herein
by reference. Using a monoclonal antibody for cGMP, RIA
was performed in the Gammaflow™ automated RIA system as
disclosed in Brooker et al., Science 191:270-276 (1976),
25 which is incorporated herein by reference. Standard
stock solutions of cGMP (20 μM) were prepared in 0.1 N
HCl and the absorbance of the solution was routinely
monitored spectrophotometrically (Shimadzu, UV 160U).
Standard dilutions (0.63-80 nM) were made from stock

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solution. The HCl extract containing cGMP was used for RIA directly. Results are summarized in Table V below and demonstrate that EX-2 can inhibit inducible nitric oxide synthase, thereby reducing levels of NO production

5   and the negative effects associated therewith, such as diabetes or glomerulonephritis.

B.   Induced Diabetes:

Male Sprague-Dawley rats were given 65 mg/kg of streptozotocin (STZ) intravenously through the tall vein.

10 Rats were divided into two groups. The first group was administered EX-2, 150 μg/kg every 8 hr for 72 hr and the second group was a control. Blood samples were withdrawn under ether anesthesia every 8 hr for the first 72 hr, then every 12 hr or 24 hr until the end of the

15 observation period. Plasma samples were assayed for glucose using a glucose assay kit (Trinder, Sigma Chemical Co.). As shown in Table VI below EX-2 effect a 72% reduction in blood glucose levels.

C.   Heritable Diabetes:

20   Male Zucker rats are a well established rat model for heritable non-insulin dependent diabetes mellitus (NIDDM), as described, for example, by Ward et al., Ann. Clin. Lab. Sci., 24:266-277, (1994), which is incorporated herein by reference. Eighteen male Zucker

25 rats were used to study the effects of EX-2 on NIDDM, a chronic disease. The rats were placed into two groups and received either saline or 25 μg/kg EX-2 every 8 hr

for approximately three months. Blood samples were
collected by tail clip and urine samples were collected
by placing animals in metabolic cages fro 24 hr. Plasma
insulin was assayed using an RIA kit (Amersham Life

5   Sciences, Arlington Heights, IL). Urine protein was
assayed for using a commercial protein assay kit
(Coomassie Plus, Pierce, Rockford, IL). Body weighed
measurements were made with a Mettler Toledo top loader
balance. As shown in Table VI, EX-2 effected a 39%

10 reduction in insulin, a 87% reduction in urine protein,
and approximately 100 gram decrease in body weight (32%
reduction; see Example XX).

TABLE VI

15   **DIABETES AND GLOMERULONEPHRITIS**

Model/Assay	Measure	% Reduction *
iNOS	cGMP (pmol/100 mg/15 min)	60%
STZ Diabetes	Blood Glucose (mg/dl)	72%
20 Zucker NIDDM Diabetes	Insulin (ng/ml)	39%
	Urine Protein (mg/24 hr)	87%
	Body Weight (grams)	32%
Glomerulonephritis	Urine Albumin (mg/day)	62%

25   * Percent reduction of increases in measures that were
     induced by Model/Assay by administration of EX-2.

D. Glomerulonephritis:

Male Sprague-Dawley rats (weight 194-248 g)
were immunized by subcutaneous injection of 1 mg normal
rabbit IgG (Sigma Chemical Co.) in Freund's complete

5   adjuvant (Sigma Chemical Co.). EX-2 was administered 3
times per day (100 μg/kg per injection) throughout the
duration of the experiment. Seven days later, the rats
were injected with 1.5 ml of nephrotoxic serum iv via
dorsal penile vein. Urine, plasma, and serum were

10 collected before injection of nephrotoxic serum at 4, 6,
24, 48, and 96 hr thereafter. All rats developed acute
glomerulonephritis with albuminuria that increased
progressively from 4 to 96 hr, as demonstrated by 24 hr
albumin excretion. Albuminuria was quantified by

15 immunoelectrophoresis. The results are summarized below
in Table VI and show that a cytokine regulatory agent of
the present invention attenuates the increase in albumin
levels seen in glomeruleronephritis, which is an
indication of improvement in the disease state.

20   In summary, the above data demonstrate that a
cytokine regulatory agent of the present invention
inhibits inducible nitric oxide synthase levels, thereby
reducing the production of NO. The cytokine regulatory
agents are also useful for treating particular diseases

25 in which nitric oxide contributes to tissue damage such
as diabetes, glomerulonephritis, atherosclerosis and
multiple sclerosis.

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Example XVII

Increasing Levels of IL-10 in Mice

This example describes the effectiveness of
EX-2 in increasing the levels of IL-10 in mammals using
5   the increase in mouse plasma subsequent to EX-2
administration as an illustrative example.

Figure 2 shows the IL-10 inducing effect of
EX-2, also designated, in mouse plasma. EX-2 was
administered intraperitoneally to 19 mice in doses
10   ranging from 1.0 ug/mouse to 1000.0 ug/mouse. Results
showed a dose dependent increase in IL-10 levels ranging
from 5.0 pg/ml to 23.0 pg/ml.

Example XVIII

15   ### Increasing Levels of IL-10 in Humans

This example shows the cytokine enhancing
effect of the cytokine regulatory agent EX-2 in a human
subject.

EX-2 was administered by intravenous infusion
20   to eight healthy male volunteers in doses of either 200
or 500 ug/kg. Plasma IL-10 levels increased in two of
four volunteers after administration at 200 ug/kg EX-2
(Figure 3a) and in four of four volunteers after
administration of 500 ug/kg EX-2 (Figure 3b). The
25   results show an 85% increase of IL-10 in subjects

receiving 200 ug/kg EX-2 and an increase of 230% in
subjects receiving 500 ug/kg EX-2.

Example XIX

Treatment of the Adverse Side Effects Caused
by Cancer Chemotherapy in Humans

This example describes the utility of a
cytokine regulatory agent such as EX-2 in ameliorating
the adverse side effects associated with the cancer
chemotherapy using cisplatin, Taxol* or Adriamycin*, which
induce and enhance the production of cytokines.

EX-2, for example, can be administered by IV
infusion to patients undergoing or about to undergo
chemotherapy. For example, a patient can be scheduled to
receive cisplatin, Taxol*, Adriamycin* or another
appropriate chemotherapeutic or radiologic treatment.
EX-2 can be administered in a dose of about between 1 and
1000 ug/kg, with a preferred infusion dose of 500 ug/kg.
Doses can be administered by continuous drip or by
intermittent bolus.

EXAMPLE XX

Reduction in Body Weight Due to
Administration of a Cytokine Regulatory Agent

This example demonstrates that administration
of a cytokine regulatory agent can cause a decrease in
the body weight of a subject.

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Adult Zucker fatty rats (fa/fa; 325-350 g) or
non-obese Zucker rats (Fa/+; 250-275 g) each were divided
into two groups. One group each of fa/fa and Fa/+ rats
received intraperitoneal (ip) injections of 250 μg/kg
5   EX-2, 3x/day, for 86 days, followed by 2x/day treatments
for an additional 29 days. The other group of fa/fa and
Fa/+ rats received saline on the same schedule.

Administration of EX-2 caused a significant
decrease in the weight gain of fa/fa and of Fa/+ rats
10   (see Figure 4). Specifically, saline treated fa/fa rats
gained about 360 g during the 105 day treatment, whereas
EX-2 treated fa/fa rats only gained 275 g, representing a
24% decrease in weight gain. Significantly, this
decrease in weight correlated to decreased epididymal fat
15   pad weight, indicating that fat, not lean muscle mass,
weight is decreased by administration of EX-2. In Fa/+
rats, the decreased weight gain was more modest, but
again correlated to fat mass rather than lean muscle
mass. These results indicate that a cytokine regulatory
20   agent can decrease weight gain in normal and obese
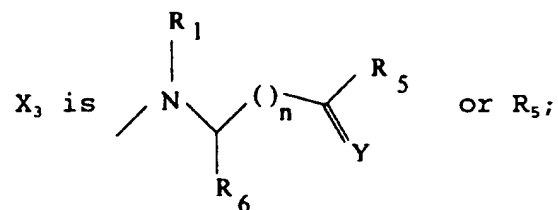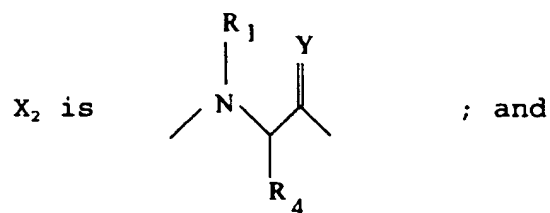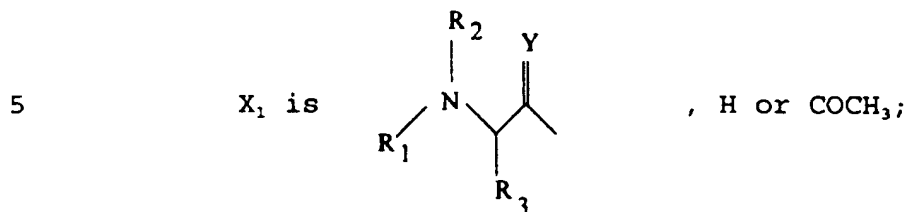subjects by decreasing the accumulation of fat.

In other experiments, fa/fa rats received
0.5-10 mg/kg EX-2, 2x/day, for 14 days (1-20 mg/day). A
decreased rate of weight gain was observed in each group.
25   Furthermore, rats treated with 20 mg EX-2/day showed
about a 10% decrease in body weight. This weight loss
correlated with increased consumption by the EX-2
treated rats as compared to the controls. These results

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indicate that a cytokine regulatory agent is useful for decreasing the body weight of a subject.

Although the invention has been described with
5   reference to the examples provided above, it should be understood that various modifications can be made by those skilled in the art without departing from the invention. Accordingly, the invention is set out in the following claims.

We claim:

　　1.　A cytokine regulatory agent, comprising:

　　X_1 - X_2 - His - (D)Phe - Arg - (D)Trp - X_3,

　　wherein:

X_1 is

, H or $COCH_3$;

X_2 is

; and

X_3 is

or R_5;

wherein Y is O, H_2 or S;

　　R_1 is H, $COCH_3$, C_2H_5, CH_2Ph, COPh, $COOCH_2Ph$, COO-t-butyl, CH_2CO-(polyethylene glycol) or A;
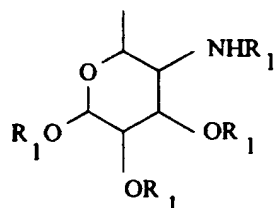
　　R_2 is H or $COCH_3$;

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R_3 is a linear or branched alkyl group having 1 to 6 carbon atoms or a cyclic alkyl group having 3 to 6 carbon atoms;

R_4 is $(CH_2)_m$-$CONH_2$, $(CH_2)_m$-$CONHR_1$ or $(CH_2)_m$-$CONHA$;

R_5 is OH, OR_3, NH_2, SH, $NHCH_3$, $NHCH_2Ph$ or A; and

R_6 is H or R_3;

and wherein "Ph" is C_6H_5; "m" is 1, 2 or 3; "n" is 0, 1, 2 or 3; and "A" is a carbohydrate having the general formula:

2.   The peptide of claim 1, wherein the amino terminus is modified.

3.   The peptide of claim 1, wherein the carboxy terminus is modified.

4.   The peptide of claim 1, wherein R_1 is selected from the group consisting of C_2H_5 and CH_2Ph and wherein R_2 is selected from the group consisting of H and $COCH_3$.

5. The peptide of claim 1, wherein R_1 and R_2 are the same moiety, said moiety selected from the group consisting of H, C_2H_5 and CH_2Ph.
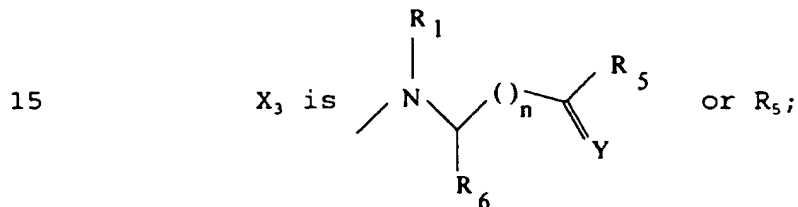
6. The peptide of claim 1, wherein X_1 is
5 selected from the group consisting of norleucine, norvaline, leucine or isoleucine.

7. The peptide of claim 1, wherein R_5 is covalently bound to X_1, said covalent bond forming a cyclic peptide.

10   8. A cytokine regulatory agent, comprising:

$$X_4 - X_5 - (D)Phe - Arg - (D)Trp - X_3,$$

wherein:

X_4 is , H, $COCH_3$, or absent;

X_5 is His, H, or $COCH_3$; and

15   X_3 is or R_5;
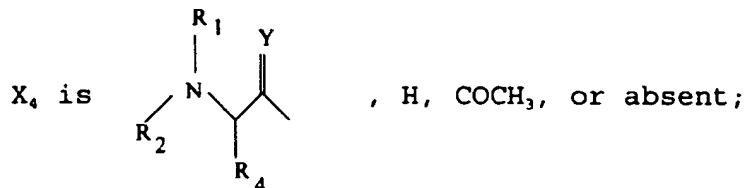
wherein Y is O, H_2 or S;

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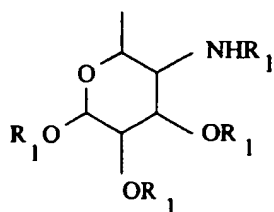R_1 is H, $COCH_3$, C_2H_5, CH_2Ph, $COPh$, $COOCH_2Ph$, COO-t-butyl, CH_2CO-(polyethylene glycol) or A;

R_2 is H or $COCH_3$;

R_4 is $(CH_2)_m$-$CONH_2$, $(CH_2)_m$-$CONHR_1$ or $(CH_2)_m$-$CONHA$;

R_5 is OH, OR_3, NH_2, SH, $NHCH_3$, $NHCH_2Ph$ or A; and

R_6 is H or R_3;

and wherein "Ph" is C_6H_5; "m" is 1, 2 or 3; "n" is 0, 1, 2 or 3; and "A" is a carbohydrate having the general formula:

9.   The peptide of claim 8, wherein the amino terminus is modified.

10.   The peptide of claim 8, wherein the carboxy terminus is modified.

11.   The peptide of claim 8, wherein R_1 is selected from the group consisting of C_2H_5 and CH_2Ph and wherein R_2 is selected from the group consisting of H and $COCH_3$.

5   12.   The peptide of claim 8, wherein R_1 and R_2 are the same moiety, said moiety selected from the group consisting of H, C_2H_5 and CH_2Ph.

13.   The peptide of claim 8, wherein R_5 is covalently bound to X_4, said covalent bond forming a

10   cyclic peptide.

14.   A cytokine regulatory agent having an amino acid sequence selected from the group consisting of:

(cyclohexyl)Gly-Gln-His-(D)Phe-Arg-(D)Trp-Gly;

15   His-(D)Phe-Arg-(D)Trp-Gly;

His-(D)Phe-Arg-(D)Trp;

(D)Phe-Arg-(D)Trp; and

His-(D)Phe-Arg-{(D)Trp(CH$_2$)}-(NAc)Gly.

15.   A peptide of claim 14, wherein the carboxy

20   terminus is modified by amidation.

16.   A peptide of claim 14, wherein the amino terminus of said peptide is acetylated.

17.   A cytokine regulatory agent, having the amino acid sequence cyclo(His-(D)Phe-Arg-(D)Trp).

18. A method of regulating cytokine activity in a subject having a condition characterized by altered or aberrant cytokine activity, comprising administering to the subject an effective amount of the cytokine

5   regulatory agent of claim 1.

19. The method of claim 18, wherein said cytokine regulatory agent enhances said cytokine activity.

10   20. The method of claim 19, wherein said cytokine activity is IL-10 activity.

21. The method of claim 19, wherein said condition is an inflammatory response.

22. The method of claim 19, wherein said

15   condition is cachexia.

23. The method of claim 19, wherein said condition is a patho-immunogenic response.

24. The method of claim 19, wherein said condition is adult respiratory distress syndrome.

20   25. A method of reducing the effects of disuse deconditioning in a subject, comprising administering to the subject an effective amount of the cytokine regulatory agent of claim 1.

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26.   A method of protecting an organ of a
subject against organ damage, comprising administering to
the subject an effective amount of the cytokine
regulatory agent of claim 1.

5   27.   The method of claim 26, wherein the organ
damage is the result of a condition selected from the
group consisting of organ transplant, the administration
of an immunosuppressive agent, and ischemia-reperfusion.

               28.   The method of claim 27, wherein the
10   immunosuppressive agent is cyclosporine.

               29.   A method of ameliorating an adverse side
effect of cancer chemotherapy in a subject, comprising
administering to the subject an effective amount of a
cytokine regulatory agent of claim 1.

15   30.   A method of claim 29, wherein the adverse
effect is the nephrotoxic effect of a cancer
chemotherapeutic agent.

               31.   The method of claim 30, wherein the cancer
chemotherapeutic agent is selected from the group
20   consisting of cisplatin, Taxol®, and Adriamycin®.

               32.   A method of treating a subject having a
disease mediated by nitric oxide and cytokines,
comprising administering to the subject an effective
amount of the cytokine regulatory agent of claim 1.

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33. The method of claim 32, wherein the disease is selected from the group consisting of diabetes and glomerulonephritis.

5   34. A method of decreasing the body weight of a subject, comprising administering to the subject an effective amount of the cytokine regulatory agent of claim 1.

35. A method of increasing the oxygen consumption of a subject, comprising administering to the
10 subject an effective amount of the cytokine regulatory agent of claim 1.

36. A method of regulating cytokine activity in a subject having a condition characterized by altered or aberrant cytokine activity, comprising administering
15 to the subject an effective amount of the cytokine regulatory agent of claim 8.

37. The method of claim 36, wherein said cytokine regulatory agent enhances said cytokine activity.

20   38. The method of claim 37, wherein said cytokine activity is IL-10 activity.

39. The method of claim 37, wherein said condition is an inflammatory response.

40.   The method of claim 37, wherein said
condition is cachexia.

41.   The method of claim 37, wherein said
condition is a patho-immunogenic disease.

5   42.   The method of claim 37, wherein said
condition is adult respiratory distress syndrome.

43.   A method of reducing the effects of disuse
deconditioning in a subject, comprising administering to
the subject an effective amount of the cytokine
10   regulatory agent of claim 8.

44.   A method of protecting an organ of a
subject against organ damage, comprising administering to
the subject an effective amount of the cytokine
regulatory agent of claim 8.

15   45.   The method of claim 44, wherein the organ
damage is the result of a condition selected from the
group consisting of organ transplant, the administration
of an immunosuppressive agent, and ischemia-reperfusion.

46.   The method of claim 45, wherein the
20   immunosuppressive agent is cyclosporine.

47.   A method of ameliorating an adverse side
effect of cancer chemotherapy in a subject, comprising
administering to the subject an effective amount of a
cytokine regulatory agent of claim 8.

48.   A method of claim 47, wherein said adverse side effect is nephrotoxicity.

49.   The method of claim 47, wherein the chemotherapy comprises administering a chemotherapeutic
5   agent selected from the group consisting of cisplatin, Taxol*, and Adriamycin*.

50.   A method of treating a subject having a disease mediated by nitric oxide and cytokines, comprising administering to the subject an effective
10   amount of the cytokine regulatory agent of claim 8.

51.   The method of claim 50, wherein the disease is selected from the group consisting of diabetes and glomerulonephritis.

15   52.   A method of decreasing the body weight of a subject, comprising administering to the subject an effective amount of the cytokine regulatory agent of claim 8.

53.   A method of increasing the oxygen
20   consumption of a subject, comprising administering to the subject an effective amount of the cytokine regulatory agent of claim 8.

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54.   A method of regulating cytokine activity
in a subject having a condition characterized by altered
or aberrant cytokine activity, comprising administering
to the subject an effective amount of a cytokine

5   regulatory agent of claim 14.

55.   A method of regulating cytokine activity
in a subject having a condition characterized by altered
or aberrant cytokine activity, comprising administering
to the subject an effective amount of the cytokine

10   regulatory agent of claim 17.

56.   A method of reducing the effects of disuse
deconditioning in a subject, comprising administering to
the subject an effective amount of the cytokine
regulatory agent having the sequence:

15   Ac-Nle-Gln-His-(D)Phe-Arg-(D)Trp-Gly-NH$_2$.

57.   A method of protecting an organ of a
subject against organ damage, comprising administering to
the subject an effective amount of the cytokine
regulatory agent having the sequence:

20   Ac-Nle-Gln-His-(D)Phe-Arg-(D)Trp-Gly-NH$_2$.

58.   A method of reducing an adverse side
effect of cancer chemotherapy in a subject, comprising
administering to said subject an effective amount of a
cytokine regulatory agent having the sequence:

25   Ac-Nle-Gln-His-(D)Phe-Arg-(D)Trp-Gly-NH$_2$.